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(54) Titre : ANTICORPS PRESENTANT UNE SPECIFITE DE TYPE RECEPTEUR DES LYMPHOCYTES T, UNE AFFINITE ENCORE SUPERIEURE, ET UTILISATION DE CELUI-CI DANS LA DETECTION ET LE TRAITEMENT DU CANCER, D'INFECTIONS VIRALES ET DE MALADIES AUTO-IMMUNES

(54) Title: ANTIBODY HAVING A T-CELL RECEPTOR-LIKE SPECIFICITY, YET HIGHER AFFINITY, AND THE USE OF SAME IN THE DETECTION AND TREATMENT OF CANCER, VIRAL INFECTION AND AUTOIMMUNE DISEASE

(57) Abrégé/Abstract:

An isolated molecule which comprises an antibody specifically bindable with a binding affinity below 20 nanomolar, preferably below 10 nanomolar, to a human major histocompatibility complex (MHC) class I being complexed with a HLA-restricted antigen and optionally further comprises an identifiable or therapeutic moiety conjugated to the antibody.

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**WO 03/068201 A3**

(54) Title: HIGH AFFINITY ANTIBODY HAVING A TCR-LIKE SPECIFICITY AND USE IN DETECTION AND TREATMENT

(57) Abstract: An isolated molecule which comprises an antibody specifically bindable with a binding affinity below 20 nanomolar, preferably below 10 nanomolar, to a human major histocompatibility complex (MHC) class I being complexed with a HLA-restricted antigen and optionally further comprises an identifiable or therapeutic moiety conjugated to the antibody.

ANTIBODY HAVING A T-CELL RECEPTOR-LIKE SPECIFICITY, YET  
HIGHER AFFINITY, AND THE USE OF SAME IN THE DETECTION AND  
TREATMENT OF CANCER, VIRAL INFECTION AND AUTOIMMUNE  
DISEASE

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FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to an antibody having a T-cell receptor specificity and higher affinity, conjugates of same with identifiable and/or therapeutic moieties, method of making the antibody and the conjugates, polynucleotides encoding the antibody and conjugates and methods of using the conjugates in the detection and treatment of cancer, viral infection and autoimmune disease.

The expression of specific peptides in complex with major histocompatibility complex (MHC) class I molecules on cells was shown to be associated with cancer and autoimmune disorders (1-3) and viral infections. In cancer, the discovery of these peptides emerged from the now well-established observation that human tumor cells often express antigens that are recognized by cytotoxic T lymphocytes (CTLs) derived from patients (1-5).

Moreover, it has been demonstrated that the immune response against the tumor is insufficient to cause tumor regression and that tumor cells can develop effective mechanisms to escape such an immune attack (6-9). Therefore, numerous approaches are being developed in the field of tumor vaccination in an attempt to augment the antitumor immune responses, including cancer peptide vaccines, autologous cancer vaccines, and the cancer-dendritic cell hybrid vaccine (7, 10, 11).

Because the specificity of the immune response is regulated and dictated by these class I MHC-peptide complexes, it should be possible to use these very specific and unique molecular cell-surface markers as targets to eliminate the cancer cells, while sparing the normal cells. A similar approach can be undertaken to eradicate viral infected cells and cells presenting targets for autoimmune attack. Thus, it would be very desirable to devise new molecules in a soluble form that will mimic the fine, unique specificity of the T-cell



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antigen receptor (TCR) to the cancer/viral/autoimmune-associated MHC-peptide complexes.

One promising approach is to generate recombinant antibodies that will bind the MHC-peptide complex expressed on the cancer cells surface with the same specificity as the TCR. These unique antibodies can subsequently be armed with an effector cytotoxic moiety such as a radioisotope, a cytotoxic drug, or a toxin. For example, antibodies that target cancer cells were genetically fused to powerful toxins originating from both plants and bacteria, thus generating molecules termed recombinant immunotoxins (12).

Antibodies with the MHC-restricted specificity of T cells are rare and have been difficult to generate by conventional hybridoma techniques because B cells are not educated to be self-MHC-restricted (13-16). The advantages of antibody phage-displayed technology makes it possible to also select large antibody repertoires for unique and rare antibodies against very defined epitopes. This has been demonstrated by the ability to isolate by phage display a TCR-like restricted antibody to a murine class I MHC H-2K<sup>k</sup> complexed with a viral epitope (17). Evidently, this antibody, being directed at mouse MHC, is useless in the treatment and diagnosis of humans. So far, attempts made by the same group to develop a TCR-like restricted antibody to a human class I MHC have failed. More recently an antibody was isolated reactive with the melanoma antigen MAGE-A1 in a complex with HLA-A1; however this antibody exhibited a low affinity and could be used to detect the specific complexes on the surface of antigen-presenting cells only when expressed in a multimeric form on a phage and not as a soluble antibody (18).

There is thus a widely recognized need for, and it would be highly advantageous to have, a TCR-like restricted antibody to a human class I MHC devoid of the above limitations.

SUMMARY OF THE INVENTION

In recent years, many cancer-associated, viral and autoimmune associated MHC-restricted peptides have been isolated and because of their highly restricted fine specificity, they are desirable targets for novel approaches in immunotherapy and immunodiagnosis. Antibodies that are able to recognize cancer-associated, viral and autoimmune associated MHC-peptide complexes, with the same specificity as the T-cell antigen receptor, would be valuable reagents for studying antigen presentation by tumor cells, viral infected cells and autoimmune related cells, for visualizing MHC-peptide complexes on such cells, and eventually for developing new targeting agents for cancer, viral and autoimmune immunotherapy and immunodiagnosis.

While reducing the present invention to practice, and in order to generate exemplary molecules with such a unique, fine specificity, HLA-A2 transgenic mice were immunized with a soluble single-chain HLA-A2, complexed with a common antigenic T cell HLA-A2-restricted epitope derived from the melanoma differentiation antigen gp100. Using phage display, a high affinity recombinant scFv antibody that exhibits a characteristic TCR-like binding specificity to the gp100-derived epitope, yet unlike TCRs, it does so with an affinity in the nanomolar range was isolated. The TCR-like antibody recognizes the native MHC-peptide complex expressed on the surface of antigen-presenting cells. Moreover, when fused to a very potent cytotoxic effector molecule in the form of a truncated bacterial toxin, it was able to specifically kill antigen-presenting cells in a peptide-dependent manner and with TCR-like specificity. These results demonstrate, for the first time, the ability to isolate high-affinity human recombinant antibodies with the antigen-specific, MHC-restricted specificity of T cells directed toward human cancer T-cell epitopes. The selected TCR-like antibodies are useful for monitoring and visualizing the expression of specific MHC-peptide complexes on the surface of tumor cells, other cells presenting antigens, and lymphoid



tissues, as well as for developing a new family of targeting agents for immunotherapy.

Hence, according to one aspect of the present invention there is provided an isolated molecule comprising an antibody specifically bindable with a binding affinity below 20 nanomolar, preferably below 10 nanomolar, to a human major histocompatibility complex (MHC) class I being complexed with a HLA-restricted antigen.

According to further features in preferred embodiments of the invention described below, the isolated molecule further comprising an identifiable moiety being conjugated to the antibody.

According to still further features in the described preferred embodiments the isolated molecule further comprising a therapeutic moiety being conjugated to the antibody.

In one example, the antibody is a single chain antibody having an amino acid sequence as set forth in SEQ ID NO:9.

According to another aspect of the present invention there is provided a pharmaceutical composition comprising a therapeutically effective amount of a molecule which comprises an antibody specifically bindable with a binding affinity below 20 nanomolar to a human major histocompatibility complex (MHC) class I being complexed with a HLA-restricted antigen, the molecule further comprises a therapeutic moiety being conjugated to the antibody. Preferably, the pharmaceutical composition further comprising a pharmaceutically acceptable carrier.

According to yet another aspect of the present invention there is provided a diagnostic composition comprising a molecule which comprises an antibody specifically bindable with a binding affinity below 20 nanomolar to a human major histocompatibility complex (MHC) class I being complexed with a HLA-restricted antigen, the molecule further comprises an identifiable moiety being conjugated to the antibody.

According to still another aspect of the present invention there is provided an isolated molecule comprising a first polynucleotide encoding an antibody specifically bindable with a binding affinity below 20 nanomolar to a human major histocompatibility complex (MHC) class I being complexed with a HLA-restricted antigen.

In one example, the first polynucleotide encodes a protein having an amino acid sequence as set forth in SEQ ID NO:9. In a specific example, the first polynucleotide has a nucleic acid sequence as set forth in SEQ ID NO:8.

According to further features in preferred embodiments of the invention described below, the isolated molecule further comprising a second polynucleotide being linked to the first polynucleotide and encoding a therapeutic moiety.

According to alternative features in preferred embodiments of the invention described below, the isolated molecule of claim 24, further comprising a second polynucleotide being linked to the first polynucleotide and encoding an identifiable moiety.

According to still further features in the described preferred embodiments the identifiable moiety is selected from the group consisting of a member of a binding pair and a label.

According to still further features in the described preferred embodiments the member of the binding pair is an antigen.

According to still further features in the described preferred embodiments the label is selected from the group consisting of a fluorescent protein and an enzyme.

According to an additional aspect of the present invention there is provided a method of producing an antibody specifically bindable with a binding affinity below 20 nanomolar to a human major histocompatibility complex (MHC) class I being complexed with a HLA-restricted antigen, the method comprising: immunizing a genetically engineered non-human mammal having cells expressing the human major histocompatibility complex (MHC)



class I with a soluble form of a MHC class I molecule being complexed with the HLA-restricted antigen; isolating mRNA molecules from antibody producing cells of the non-human mammal; producing a phage display library displaying protein molecules encoded by the mRNA molecules; and isolating  
5 at least one phage from the phage display library, the at least one phage displaying the antibody specifically bindable with the affinity below 10 nanomolar to the human major histocompatibility complex (MHC) class I being complexed with the HLA-restricted antigen.

According to still further features in the described preferred  
10 embodiments the non-human mammal is devoid of self MHC class I molecules.

According to still further features in the described preferred embodiments the HLA-restricted antigen is a tumor HLA-restricted antigen.

According to still further features in the described preferred embodiments the HLA-restricted antigen is a viral HLA-restricted antigen.

15 According to still further features in the described preferred embodiments the HLA-restricted antigen is an autoimmune HLA-restricted antigen.

According to still further features in the described preferred  
20 embodiments the soluble form of a MHC class I molecule is a single chain MHC class I polypeptide including a functional human  $\beta$ -2 microglobulin amino acid sequence directly or indirectly covalently linked to a functional human MHC class I heavy chain amino acid sequence.

According to still an additional aspect of the present invention there is provided a method of treating a cancer, the method comprising administering to  
25 a subject in need thereof a therapeutically effective amount of a molecule which comprises an antibody specifically bindable with a binding affinity below 20 nanomolar to a human major histocompatibility complex (MHC) class I being complexed with a tumor HLA-restricted antigen characterizing the cancer, the molecule further comprises a therapeutic moiety being conjugated to the



antibody, the MHC class I molecule being selected matching to the endogenous MHC class I of the subject.

According to yet an additional aspect of the present invention there is provided a method of treating a viral infection, the method comprising  
5 administering to a subject in need thereof a therapeutically effective amount of a molecule which comprises an antibody specifically bindable with a binding affinity below 20 nanomolar to a human major histocompatibility complex (MHC) class I being complexed with a viral HLA-restricted antigen characterizing a virus causative of the viral infection, the molecule further  
10 comprises a therapeutic moiety being conjugated to the antibody, the MHC class I molecule being selected matching to the endogenous MHC class I of the subject.

According to yet an additional aspect of the present invention there is provided a method of treating an autoimmune disease, the method comprising  
15 administering to a subject in need thereof a therapeutically effective amount of a molecule which comprises an antibody specifically bindable with a binding affinity below 20 nanomolar to a human major histocompatibility complex (MHC) class I being complexed with an autoimmune HLA-restricted antigen, the molecule further comprises a therapeutic moiety being conjugated to the  
20 antibody, the MHC class I molecule being selected matching to the endogenous MHC class I of the subject.

According to further features in preferred embodiments of the invention described below, the MHC class I molecule is selected from the group consisting of HLA-A2, HLA-A1, HLA-A3, HLA-A24, HLA-A28, HLA-A31,  
25 HLA-A33, HLA-A34, HLA-B7, HLA-B45 and HLA-Cw8.

According to still further features in the described preferred embodiments the therapeutic moiety is selected from the group consisting of a cytotoxic moiety, a toxic moiety, a cytokine moiety and a bi-specific antibody moiety.

According to another aspect of the present invention there is provided a method of making an immunotoxin, the method comprising ligating a first polynucleotide encoding an antibody specifically bindable with a binding affinity below 20 nanomolar to a human major histocompatibility complex (MHC) class I being complexed with a HLA-restricted antigen in frame with a second polynucleotide encoding a toxin moiety, so as to obtain a ligated polynucleotide and expressing the ligated polynucleotide in an expression system so as to obtain the immunotoxin.

According to another aspect of the present invention there is provided a method of making an immunolabel, the method comprising ligating a first polynucleotide encoding an antibody specifically bindable with a binding affinity below 20 nanomolar to a human major histocompatibility complex (MHC) class I being complexed with a HLA-restricted antigen in frame with a second polynucleotide encoding an identifiable moiety, so as to obtain a ligated polynucleotide and expressing the ligated polynucleotide in an expression system so as to obtain the immunolabel.

According to another aspect of the present invention there is provided a method of detecting the presence and/or level of antigen presenting cells presenting a HLA-restricted antigen in a sample of cells, the method comprising interacting cells of the sample with an antibody specifically bindable with a binding affinity below 20 nanomolar to a human major histocompatibility complex (MHC) class I being complexed with a HLA-restricted antigen; and monitoring the interaction, thereby detecting the presence and/or level of the antigen presenting cells presenting the HLA-restricted antigen.

Depending on the application, the HLA-restricted antigen is selected from the group consisting of a tumor HLA-restricted antigen, a viral HLA-restricted antigen and an autoimmune HLA-restricted antigen.

The present invention successfully addresses the shortcomings of the presently known configurations by providing an antibody having a T-cell



receptor specificity and high affinity to its antigen, and the use thereof in immunotherapy and immunodiagnosis.

### BRIEF DESCRIPTION OF THE DRAWINGS

5 The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred  
10 embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art  
15 how the several forms of the invention may be embodied in practice.

In the drawings:

FIGs. 1A-B show bar graphs demonstrating polyclonal phage ELISA on recombinant scHLA-A2-peptide complexes. Plates were coated with the indicated scMHC-peptide complex as described in the Examples section that  
20 follows. Shown is the binding of the polyclonal phage population of the initial library (L) or phages eluted after each round of panning (I-IV). Figure 1A - phages from the pCANTAB scFv library; Figure 1B - phages from the scFv-CBD library. Binding specificity studies were performed by ELISA using biotinylated scMHC-peptide complexes. ELISA plates (Falcon) were coated  
25 overnight with BSA-biotin (1µg/well), washed, incubated (1 hr, RT) with streptavidin (1 µg/well), washed again extensively and further incubated (1 hr, RT) with 0.5 µg of MHC/peptide complexes. Plates were blocked with PBS/2% milk (30 min, RT), incubated with phage clones (~10<sup>9</sup> phages/well, 1 hr, RT) or 0.5-1 µg of soluble scFv or scFv-PE38, and afterwards washed with  
30 1:1000 HRP-conjugated/anti-M13, anti-*myc* antibody or anti-PE antibody,

respectively. The HLA-A2-restricted peptides used for specificity studies are gp100 (154): KTWGQYWQV (SEQ ID NO:1); gp100 (209): IMDQVPFSV (SEQ ID NO:2); gp100 (280): YLEPGPVTV (SEQ ID NO:3); MUC1: LLLTVLTVL (SEQ ID NO:4); HTLV-1 (TAX): LLFGYPVYV (SEQ ID NO:5); hTERT (540): ILAKFLHWL (SEQ ID NO:6); hTERT (865): RLVDDFLLV (SEQ ID NO:7).

FIGs. 2A-B show bar graphs demonstrating differential binding of monoclonal phage clones to scHLA-A2/gp100 complexes. Monoclonal phage were tested for binding to immobilized scHLA-A2 complexed with the gp100-derived epitopes. Figure 2A - G9-209M; Figure 2B - G9-280V. The assays were performed similar to as described under Figure 1 above.

FIG. 3A shows the nucleic (SEQ ID NO:8) and amino (SEQ ID NO:9) acid sequences of the antibody G1 scFv. CDRs are marked in bold, the peptide linker connecting the VH and VL domains is underlined.

FIGs. 3B-C show SDS-PAGE analysis of purified G1scFv and G1scFv-PE38. The G1 scFv gene was rescued from the phage clone by PCR and was subcloned into the phagemid vector pCANTAB6 via the *SfiI-NotI* cloning sites. A Myc and hexahistidine tags were fused to the C-terminus of the scFv gene. The scFv antibody was expressed in BL21  $\lambda$ DE3 cells as previously described (29) and purified from the periplasmic fraction by metal-ion affinity chromatography. For the expression of the G1scFv-PE38 fusion protein, the scFv gene was subcloned as an *NcoI-NotI* fragment into the plasmid pIB-NN, which encodes the translocation and ADP-ribosylation domains of PE (PE38). Expression in BL21  $\lambda$ DE3 cells, refolding from inclusion bodies, and purification of G1scFv-PE38 was performed as previously described (30).



FIG. 4 is a bar graph demonstrating the binding specificity of G1 scFv-PE38. Immunoplates were coated with various indicated scHLA-A2-peptide complexes as described and binding of the G1 scFv-PE38 to immobilized complexes was detected with anti-PE antibodies.

5 FIGs. 5A-B show plots demonstrating the binding characteristics of the TCR-like G1 scFv. 5A - Titration ELISA of purified soluble G1 scFv. Wells were coated with the MHC-peptide complexes as described in the Examples section that follows. 5B - Competitive binding analysis of the ability of purified G1 scFv-PE38 to inhibit the binding of <sup>125</sup>I-labeled G1 scFv-PE38 to the  
10 HLA-A2/G9-209M complex. The apparent binding affinity of the recombinant scFv was determined as the concentration of competitor (soluble purified G1scFv-PE38) required for 50 % inhibition of the binding of the <sup>125</sup>I-labeled tracer. Flexible ELISA plates were coated with BSA-biotin and scMHC-peptide complexes (10 µg in 100 µl) were immobilized as previously  
15 described. The recombinant G1scFv-PE38 was labeled with [<sup>125</sup>I] using the Bolton-Hunter reagent. Labeled protein was added to wells as a tracer (3-5x10<sup>5</sup> CPM/well) in the presence of increasing concentrations of cold G1scFv-PE38 as a competitor and incubated at room temperature for 1 hr in PBS. The plates were washed thoroughly with PBS and the bound radioactivity was determined  
20 by a gamma counter. The apparent affinity of the G1scFv-PE38 was determined by extrapolating the concentration of a competitor necessary to achieve 50 % inhibition of [<sup>125</sup>I]-labeled G1scFv-PE38 binding to the immobilized scMHC-peptide complex. Non-specific binding was determined by adding a 20-40-fold excess of unlabeled Fab.

25 FIGs. 6A-C show plots demonstrating the binding of G1 scFv to APCs. RMA-S-HHD or JY cells were loaded with the indicated HLA-A2-restricted peptides. Peptide loaded cells were then incubated with the soluble purified G1 scFv antibody. Detection of binding was with FITC-labeled anti-Myc. RMA-S-HHD cells loaded with the G9-209 and G9-280 peptides and stained  
30 together with control unloaded cells the anti-HLA antibody w6/32 (6A) or

anti-HLA-A2 antibody BB7.2 (6B) to demonstrate the stabilization/expression of HLA-A2 complexes on the surface of peptide loaded but not on peptide-unloaded cells. Cells loaded with G9-209 or G9-280 peptides were stained with G1 scFv and the differential staining is shown (6C). The B cell  
5 line RMAS-HHD transfected with a single-chain  $\beta$ 2M-HLA-A2 gene (26) or the EBV-transformed B-lymphoblast JY cells ( $10^6$  cells) were washed twice with serum-free RPMI and incubated overnight at 26 °C or 37 °C, respectively, in medium containing 100  $\mu$ M of the peptide. The APCs were subsequently incubated at 37 °C for 2-3 hours to stabilize cell surface expression of  
10 MHC-peptide complexes followed by incubation with recombinant scFv (10-50  $\mu$ g/ml, 60-90 minutes, 4 °C) in 100  $\mu$ l. The cells were then washed, incubated with FITC-labeled anti-Myc antibody (30-45 minutes, 4 °C), and finally washed and analyzed by a FACStar\* flow cytometer (Becton Dickinson). Melanoma cells were pulsed at 37 °C with 1-10  $\mu$ M of peptide and then stained  
15 with the scFv as described herein.

FIGs. 7A-C show plots and a bar graph demonstrating the cytotoxic activity of G1 scFv-PE38 on peptide-loaded APCs. RMAS-HHD (7A) or JY (7B) cells were loaded with the HLA-A2-restricted peptides as indicated, followed by incubation with increasing concentrations of G1 scFv-PE38.  
20 Protein synthesis was determined by incorporation of  $^3$ H-Leucine into cellular proteins. In (7C) excess (0.15-0.25 mg/ml) of the indicated scHLA-A2-peptide complex was added to the wells before the addition of G1 scFv-PE38 (25-50 ng/ml). RMAS-HHD and JY APCs were loaded with the G9-209 peptide and control peptides as described above. Peptide-loaded cells were subsequently  
25 incubated with increasing concentrations of G1scFv-PE38 and the inhibition of protein synthesis was determined by measuring the uptake of  $^3$ H-Leucine into cellular proteins, as previously described (30).  $IC_{50}$  was determined as the concentration of G1scFv-PE38 required to inhibit protein synthesis by 50 %. In competition assays, excesses of specific and non-specific HLA-A2-peptide

\*Trade-mark



complexes (35-50  $\mu\text{g}/\text{well}$ ) were added to wells 15 minutes before the addition of G1scFv-PE38.

### DESCRIPTION OF THE PREFERRED EMBODIMENTS

5 The present invention is of an antibody having a T-cell receptor specificity yet far higher affinity, conjugates of same with identifiable and/or therapeutic moieties, so as to generate immunotoxins and immunolabels, method of making the antibody and the conjugates, polynucleotides encoding the antibody and the conjugates and methods of using the conjugates in the  
10 detection and treatment of cancer, viral infection and autoimmune disease.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details  
15 set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

20 The immune system is controlled and regulated by the T-cell receptor (TCR), which specifically recognizes peptide/Major histocompatibility complex (MHC) molecules.

The advent of the application of recombinant class I MHC-peptide complexes and their tetrameric arrays now enables to detect and study rare  
25 populations of antigen-specific T cells (25, 35, 36). However, fundamental questions in immunology in general, and in tumor immunology in particular, regarding antigen presentation are still open because of the lack of reagents that will enable phenotypic analysis of antigen (MHC-peptide) presentation, the other side of the coin to MHC-peptide-TCR interactions. One way to generate  
30 such reagents is by making TCR-like antibodies; however, only a few

publications have reported the generation of self-MHC-restricted antibodies by conventional means such as the hybridoma technology (13-16). The major reason for these past difficulties may be found in the molecular nature and the resolved structures of MHC-peptide complexes. More specifically, the peptides  
5 are deeply buried inside the MHC-binding groove and therefore they are presented as extended mosaics of peptide residues intermingled with the MHC residues. It has been shown that no more than 100-300 Å<sup>2</sup> of class I MHC-bound peptide faces outwards and thus is available for direct recognition, whereas antibodies recognizing protein molecules engage about 800 Å<sup>2</sup> of their  
10 ligand (17). Thus, when generating TCR-like antibodies, these molecules will presumably recognize the peptide but will also have to be dominated by the MHC.

Until now, antibodies with TCR-like specificity have been generated against murine MHC-peptide complexes employing various strategies of  
15 immunization (17). Recently, a large human Fab library was used to select for HLA-A1-MAGE-A1-specific binding antibodies (18). One specific clone, G8, was selected which exhibited TCR-like specificity but revealed a relatively low affinity of 250 nM.

While reducing the present invention to practice, the ability to select  
20 from an immune repertoire of murine scFv fragments a high affinity antibody directed toward a human T-cell epitope was demonstrated.

According to an aspect of the present invention there is provided a method of producing an antibody specifically bindable with a binding affinity below 20 nanomolar to a human major histocompatibility complex (MHC) class  
25 I which is complexed with a HLA-restricted antigen. The method according to this aspect of the invention is effected by (i) immunizing a genetically engineered non-human mammal having cells expressing the human major histocompatibility complex (MHC) class I, with a soluble form of a MHC class I molecule being complexed with the HLA-restricted antigen; (ii) isolating  
30 mRNA molecules from antibody producing cells, such as splenocytes, of the



non-human mammal; (iii) producing a phage display library displaying protein molecules encoded by the mRNA molecules; and (iv) isolating at least one phage from the phage display library, the at least one phage displaying the antibody specifically bindable with the affinity below 10 nanomolar to the human major histocompatibility complex (MHC) class I being complexed with the HLA-restricted antigen. The genetic material of the phage isolate is then used to prepare a single chain antibody or other forms of antibodies as is further described herein below. The genetic material of the phage isolate is then used to prepare a single chain antibody or other forms of antibodies as is further described hereinbelow and which are conjugated to identifiable or therapeutic moieties. Preferably, the non-human mammal is devoid of self MHC class I molecules. Still preferably, the soluble form of a MHC class I molecule is a single chain MHC class I polypeptide including a functional human  $\beta$ -2 microglobulin amino acid sequence directly or indirectly covalently linked to a functional human MHC class I heavy chain amino acid sequence.

Hence, according to another aspect of the present invention there is provided an isolated molecule comprising an antibody specifically bindable with a binding affinity below 20 nanomolar to a human major histocompatibility complex (MHC) class I complexed with a HLA-restricted antigen. Such an antibody has a T-cell receptor specificity, yet far higher affinity. In one, non-limiting example, the antibody is a single chain antibody having an amino acid sequence as set forth in SEQ ID NO:9, encoded, for example, by the polynucleotide as set forth in SEQ ID NO:8.

Once a polynucleotide encoding an antibody having a T-cell receptor specificity as herein described is cloned, it can be modified in one of many ways in order to produce a spectrum of related-products.

In one example, the polynucleotide encoding an antibody having a T-cell receptor specificity is ligated with a second polynucleotide encoding an identifiable moiety, so as to produce an antibody having a T-cell receptor specificity conjugated to the identifiable moiety, an immunolabel. Such a

conjugate or immunolabel can be used in a method of detecting the presence and/or level of antigen presenting cells presenting a HLA-restricted antigen in a sample of cells and serve for diagnosis of cancer, viral infection or autoimmune disease. As used herein, the phrase "antigen presenting cell" includes all cells  
5 expressing MHC, class I molecules on their surface, and which are capable of presenting HLA-restricted antigens. An antigen presenting cell can be a cancer cell, a cell of the immune system, or any other cell expressing MHC, class I molecules on its surface.

Hence, according to another aspect of the present invention there is  
10 provided a method of making an immunolabel, the method comprising ligating a first polynucleotide encoding an antibody specifically bindable with a binding affinity below 20 nanomolar to a human major histocompatibility complex (MHC) class I being complexed with a HLA-restricted antigen in frame with a second polynucleotide encoding an identifiable moiety, so as to obtain a ligated  
15 polynucleotide and expressing the ligated polynucleotide in an expression system so as to obtain the immunolabel.

And, according to yet another aspect of the present invention there is provided a method of detecting the presence and/or level of antigen presenting cells presenting a HLA-restricted antigen in a sample of cells, the method  
20 comprising interacting cells of said sample with an antibody specifically bindable with a binding affinity below 20 nanomolar to a human major histocompatibility complex (MHC) class I being complexed with a HLA-restricted antigen; and monitoring said interaction, thereby detecting the presence and/or level of said antigen presenting cells presenting said  
25 HLA-restricted antigen.

Depending on the application, the HLA-restricted antigen can be a tumor HLA-restricted antigen, a viral HLA-restricted antigen and an autoimmune HLA-restricted antigen, examples of which are provided hereinbelow.

According to yet another aspect of the present invention there is provided  
30 a diagnostic composition comprising a molecule which comprises an antibody



specifically bindable with a binding affinity below 20 nanomolar to a human major histocompatibility complex (MHC) class I being complexed with a HLA-restricted antigen, the molecule further comprises an identifiable moiety being conjugated to the antibody.

5           The identifiable moiety can be a member of a binding pair, which is identifiable via its interaction with an additional member of the binding pair, and a label which is directly visualized. In one example, the member of the binding pair is an antigen which is identified by a corresponding labeled antibody. In one example, the label is a fluorescent protein or an enzyme  
10           producing a colorimetric reaction.

          In another example, the polynucleotide encoding an antibody having a T-cell receptor specificity is ligated with a second polynucleotide encoding a therapeutic moiety, so as to produce an antibody having a T-cell receptor specificity conjugated to the therapeutic moiety. Such a conjugate or  
15           immunotoxin can be used in a method of treating cancer, viral infection or autoimmune disease.

          Hence, according to another aspect of the present invention there is provided a method of making an immunotoxin, the method comprising ligating a first polynucleotide encoding an antibody specifically bindable with a binding  
20           affinity below 20 nanomolar to a human major histocompatibility complex (MHC) class I being complexed with a HLA-restricted antigen in frame with a second polynucleotide encoding a toxin moiety, so as to obtain a ligated polynucleotide and expressing said ligated polynucleotide in an expression system so as to obtain said immunotoxin.

25           The immunotoxin can be used in any one of the following therapeutic protocols:

(i)       A method of treating a cancer, the method comprising administering to a subject in need thereof a therapeutically effective amount of a molecule which comprises an antibody specifically bindable with a binding  
30           affinity below 20 nanomolar to a human major histocompatibility complex

(MHC) class I being complexed with a tumor HLA-restricted antigen characterizing the cancer, the molecule further comprises a therapeutic moiety being conjugated to the antibody, the MHC class I molecule being selected matching to the endogenous MHC class I of the subject.

5 (ii) A method of treating a viral infection, the method comprising administering to a subject in need thereof a therapeutically effective amount of a molecule which comprises an antibody specifically bindable with a binding affinity below 20 nanomolar to a human major histocompatibility complex (MHC) class I being complexed with a viral HLA-restricted antigen  
10 characterizing a virus causative of the viral infection, the molecule further comprises a therapeutic moiety being conjugated to the antibody, the MHC class I molecule being selected matching to the endogenous MHC class I of the subject.

(iii) A method of treating an autoimmune disease, the method  
15 comprising administering to a subject in need thereof a therapeutically effective amount of a molecule which comprises an antibody specifically bindable with a binding affinity below 20 nanomolar to a human major histocompatibility complex (MHC) class I being complexed with an autoimmune HLA-restricted antigen, said molecule further comprises a therapeutic moiety being conjugated  
20 to said antibody, said MHC class I molecule being selected matching to the endogenous MHC class I of the subject.

According to another aspect of the present invention there is provided a pharmaceutical composition comprising a therapeutically effective amount of a molecule which comprises an antibody specifically bindable with a binding  
25 affinity below 20 nanomolar to a human major histocompatibility complex (MHC) class I being complexed with a HLA-restricted antigen, the molecule further comprises a therapeutic moiety being conjugated to the antibody. Preferably, the pharmaceutical composition further comprising a pharmaceutically acceptable carrier.



The therapeutic moiety can be, for example, a cytotoxic moiety, a toxic moiety, a cytokine moiety and a bi-specific antibody moiety, examples of which are provided hereinbelow.

In all applications the MHC class I can be, for example, HLA-A2,  
5 HLA-A1, HLA-A3, HLA-A24, HLA-A28, HLA-A31, HLA-A33, HLA-A34,  
HLA-B7, HLA-B45 and HLA-Cw8.

The following sections provide specific examples and alternatives for each of the various aspects of the invention described herein. These examples and alternatives should not be regarded as limiting in any way, as the invention  
10 can be practiced in similar, yet somewhat different ways. These examples, however, teach one of ordinary skills in the art how to practice various alternatives and embodiments of the invention.

***Antibody:***

The term "antibody" as used to describe this invention includes intact  
15 molecules as well as functional fragments thereof, such as Fab, F(ab')<sub>2</sub>, Fv and scFv that are capable of specific, high affinity binding to a human major histocompatibility complex (MHC) class I complexed with a HLA-restricted antigen. These functional antibody fragments are defined as follows: (i) Fab, the fragment which contains a monovalent antigen-binding fragment of an  
20 antibody molecule, can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain; (ii) Fab', the fragment of an antibody molecule that can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody  
25 molecule; (iii) F(ab')<sub>2</sub>, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; F(ab')<sub>2</sub> is a dimer of two Fab' fragments held together by two disulfide bonds; (iv) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as  
30 two chains; and (c) scFv or "single chain antibody" ("SCA"), a genetically engineered molecule containing the variable region of the light chain and the

variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

Methods of making these fragments are known in the art. (See for example, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1988.

Antibody fragments according to the present invention can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* or mammalian cells (e.g. Chinese hamster ovary cell culture or other protein expression systems) of DNA encoding the fragment.

Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted  $F(ab')_2$ . This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. Pat. Nos. 4,036,945 and 4,331,647, and references contained therein.

See also Porter, R. R., *Biochem. J.*, 73: 119-126, 1959. Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

Fv fragments comprise an association of  $V_H$  and  $V_L$  chains. This association may be noncovalent, as described in Inbar et al., *Proc. Nat'l Acad. Sci. USA* 69:2659-62, 1972. Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. Preferably, the Fv fragments comprise  $V_H$  and  $V_L$  chains



connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the  $V_H$  and  $V_L$  domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by Whitlow and Filpula, *Methods*, 2: 97-105, 1991; Bird et al., *Science* 242:423-426, 1988; Pack et al., *Bio/Technology* 11:1271-77, 1993; and Ladner et al., U.S. Pat. No. 4,946,778.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick and Fry, *Methods*, 2: 106-10, 1991.

Humanized forms of non-human (e.g., murine) antibodies are chimeric molecules of immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab',  $F(ab')_2$  or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues form a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise

substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence.

5 The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992)].

10 Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed  
15 following the method of Winter and co-workers [Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature* 332:323-327 (1988); Verhoeyen et al., *Science*, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567),  
20 wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

25 Human antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, *J. Mol. Biol.*, 227:381 (1991); Marks et al., *J. Mol. Biol.*, 222:581 (1991)]. The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., *Monoclonal Antibodies and  
30 Cancer Therapy*, Alan R. Liss, p. 77 (1985) and Boerner et al., *J. Immunol.*,



147(1):86-95 (1991)]. Similarly, human can be made by introducing of human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which  
5 closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks et al., Bio/Technology 10, 779-783 (1992); Lonberg et al., Nature 368 856-859  
10 (1994); Morrison, Nature 368 812-13 (1994); Fishwild et al., Nature Biotechnology 14, 845-51 (1996); Neuberger, Nature Biotechnology 14, 826 (1996); Lonberg and Huszar, Intern. Rev. Immunol. 13 65-93 (1995).

It will be appreciated that once the CDRs of an antibody are identified, using conventional genetic engineering techniques can be used to devise  
15 expressible polynucleotides encoding any of the forms or fragments of antibodies described herein.

***A human major histocompatibility complex (MHC) class I:***

The major histocompatibility complex (MHC) is a complex of antigens encoded by a group of linked loci, which are collectively termed H-2 in the  
20 mouse and HLA in humans. The two principal classes of the MHC antigens, class I and class II, each comprise a set of cell surface glycoproteins which play a role in determining tissue type and transplant compatibility. In transplantation reactions, cytotoxic T-cells (CTLs) respond mainly against foreign class I glycoproteins, while helper T-cells respond mainly against foreign class II  
25 glycoproteins.

Major histocompatibility complex (MHC) class I molecules are expressed on the surface of nearly all cells. These molecules function in presenting peptides which are mainly derived from endogenously synthesized proteins to CD8<sup>+</sup> T cells via an interaction with the  $\alpha\beta$  T-cell receptor. The  
30 class I MHC molecule is a heterodimer composed of a 46-kDa heavy chain

which is non-covalently associated with the 12-kDa light chain  $\beta$ -2 microglobulin. In humans, there are several MHC haplotypes, such as, for example, HLA-A2, HLA-A1, HLA-A3, HLA-A24, HLA-A28, HLA-A31, HLA-A33, HLA-A34, HLA-B7, HLA-B45 and HLA-Cw8, their sequences can  
5 be found at the kabat data base.

***Peptides that bind to class I MHC molecules; HLA-restricted antigens:***

Class I, MHC-restricted peptides (also referred to herein interchangeably as HLA-restricted antigens, HLA-restricted peptides, MHC-restricted antigens)  
10 which are typically 8-10-amino acid-long, bind to the heavy chain  $\alpha$ 1- $\alpha$ 2 groove via two or three anchor residues that interact with corresponding binding pockets in the MHC molecule. The  $\beta$ -2 microglobulin chain plays an important role in MHC class I intracellular transport, peptide binding, and conformational stability. For most class I molecules, the formation of a heterodimer consisting  
15 of the MHC class I heavy chain, peptide (self or antigenic) and  $\beta$ -2 microglobulin is required for biosynthetic maturation and cell-surface expression.

Research studies performed on peptide binding to class I MHC molecules enable to define specific MHC motifs functional in displaying  
20 peptides derived from viral, tumor and self antigens that are potentially immunogenic and might elicit specific response from cytotoxic T lymphocytes (CTLs).

As used herein the term "peptide" refers to native peptides (either degradation products or synthetically synthesized peptides) and further to  
25 peptidomimetics, such as peptoids and semipeptoids which are peptide analogs, which may have, for example, modifications rendering the peptides more stable while in a body, or more immunogenic. Such modifications include, but are not limited to, cyclization, N terminus modification, C terminus modification, peptide bond modification, including, but not limited to,  $\text{CH}_2\text{-NH}$ ,  $\text{CH}_2\text{-S}$ ,  
30  $\text{CH}_2\text{-S=O}$ ,  $\text{O=C-NH}$ ,  $\text{CH}_2\text{-O}$ ,  $\text{CH}_2\text{-CH}_2$ ,  $\text{S=C-NH}$ ,  $\text{CH=CH}$  or  $\text{CF=CH}$ ,



backbone modification and residue modification. Methods for preparing peptidomimetic compounds are well known in the art and are specified in Quantitative Drug Design, C.A. Ramsden Gd., Chapter 17.2, F. Choplin Pergamon Press (1992).

5 Further detail in this respect are provided hereinunder.

As used herein in the specification and in the claims section below the term "amino acid" is understood to include the 20 naturally occurring amino acids; those amino acids often modified post-translationally *in vivo*, including for example hydroxyproline, phosphoserine and phosphothreonine; and other  
10 unusual amino acids including, but not limited to, 2-amino adipic acid, hydroxylysine, isodesmosine, nor-valine, nor-leucine and ornithine. Furthermore, the term "amino acid" includes both D- and L-amino acids. Further elaboration of the possible amino acids usable according to the present invention and examples of non-natural amino acids useful in MHC-I HLA-A2  
15 recognizable peptide antigens are given hereinunder.

Based on accumulated experimental data, it is nowadays possible to predict which of the peptides of a protein will bind to MHC, class I. The HLA-A2 MHC class I has been so far characterized better than other HLA haplotypes, yet predictive and/or sporadic data is available for all other  
20 haplotypes.

With respect to HLA-A2 binding peptides, assume the following positions (P1-P9) in a 9-mer peptide:

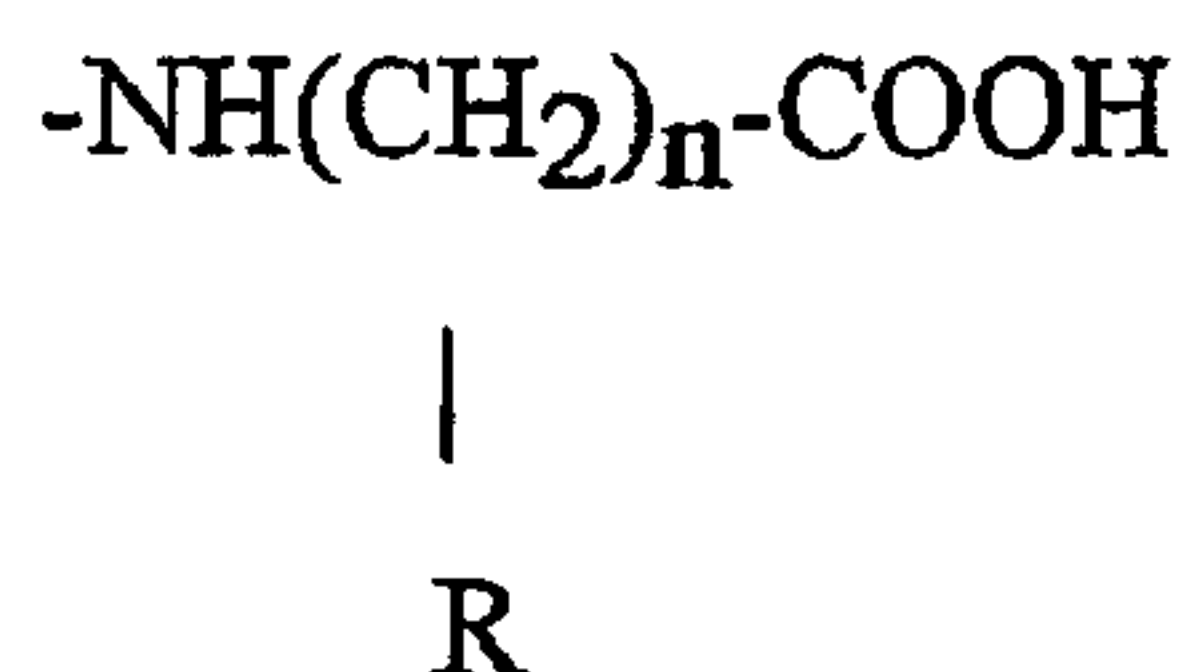
P1-P2-P3-P4-P5-P6-P7-P8-P9

The P2 and P2 positions include the anchor residues which are the main  
25 residues participating in binding to MHC molecules. Amino acid residues engaging positions P2 and P9 are hydrophilic aliphatic non-charged natural amino (examples being Ala, Val, Leu, Ile, Gln, Thr, Ser, Cys, preferably Val and Leu) or of a non-natural hydrophilic aliphatic non-charged amino acid (examples being norleucine (Nle), norvaline (Nva),  $\alpha$ -aminobutyric acid).  
30 Positions P1 and P3 are also known to include amino acid residues which

participate or assist in binding to MHC molecules, however, these positions can include any amino acids, natural or non-natural. The other positions are engaged by amino acid residues which typically do not participate in binding, rather these amino acids are presented to the immune cells. Further details relating to the binding of peptides to MHC molecules can be found in Parker, K.C., Bednarek, M.A., Coligan, J.E., Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J Immunol.152,163-175,1994., see Table V, in particular. Hence, scoring of HLA-A2.1 binding peptides can be performed using the HLA Peptide Binding Predictions software.

This software is based on accumulated data and scores every possible peptide in an analyzed protein for possible binding to MHC HLA-A2.1 according to the contribution of every amino acid in the peptide. Theoretical binding scores represent calculated half-life of the HLA-A2.1-peptide complex.

Hydrophilic aliphatic natural amino acids at P2 and P9 can be substituted by synthetic amino acids, preferably Nleu, Nval and/or  $\alpha$ -aminobutyric acid. P9 can be also substituted by aliphatic amino acids of the general formula  $-\text{HN}(\text{CH}_2)_n\text{COOH}$ , wherein  $n = 3-5$ , as well as by branched derivatives thereof, such as, but not limited to,



wherein R is, for example, methyl, ethyl or propyl, located at any one or more of the n carbons.

The amino terminal residue (position P1) can be substituted by positively charged aliphatic carboxylic acids, such as, but not limited to,  $\text{H}_2\text{N}(\text{CH}_2)_n\text{COOH}$ , wherein  $n = 2-4$  and  $\text{H}_2\text{N-C}(\text{NH})\text{-NH}(\text{CH}_2)_n\text{COOH}$ , wherein  $n = 2-3$ , as well as by hydroxy Lysine, N-methyl Lysine or ornithine (Orn). Additionally, the amino terminal residue can be substituted by enlarged



aromatic residues, such as, but not limited to,  $\text{H}_2\text{N}-(\text{C}_6\text{H}_6)-\text{CH}_2-\text{COOH}$ ,  
p-aminophenyl alanine,  $\text{H}_2\text{N}-\text{F}(\text{NH})-\text{NH}-(\text{C}_6\text{H}_6)-\text{CH}_2-\text{COOH}$ ,  
p-guanidinophenyl alanine or pyridinoalanine (Pal). These latter residues may  
form hydrogen bonding with the  $\text{OH}^-$  moieties of the Tyrosine residues at the  
5 MHC-1 N-terminal binding pocket, as well as to create, at the same time  
aromatic-aromatic interactions.

Derivatization of amino acid residues at positions P4-P8, should these  
residues have a side-chain, such as, OH, SH or  $\text{NH}_2$ , like Ser, Tyr, Lys, Cys or  
Orn, can be by alkyl, aryl, alkanoyl or aroyl. In addition, OH groups at these  
10 positions may also be derivatized by phosphorylation and/or glycosylation.  
These derivatizations have been shown in some cases to enhance the binding to  
the T cell receptor.

Longer derivatives in which the second anchor amino acid is at position  
P10 may include at P9 most L amino acids. In some cases shorter derivatives  
15 are also applicable, in which the C terminal acid serves as the second anchor  
residue.

Cyclic amino acid derivatives can engage position P4-P8, preferably  
positions P6 and P7. Cyclization can be obtained through amide bond  
formation, e.g., by incorporating Glu, Asp, Lys, Orn, di-amino butyric (Dab)  
20 acid, di-aminopropionic (Dap) acid at various positions in the chain ( $-\text{CO}-\text{NH}$   
or  $-\text{NH}-\text{CO}$  bonds). Backbone to backbone cyclization can also be obtained  
through incorporation of modified amino acids of the formulas  
 $\text{H}-\text{N}((\text{CH}_2)_n-\text{COOH})-\text{C}(\text{R})\text{H}-\text{COOH}$  or  $\text{H}-\text{N}((\text{CH}_2)_n-\text{COOH})-\text{C}(\text{R})\text{H}-\text{NH}_2$ ,  
wherein  $n = 1-4$ , and further wherein R is any natural or non-natural side chain  
25 of an amino acid.

Cyclization via formation of S-S bonds through incorporation of two Cys  
residues is also possible. Additional side-chain to side chain cyclization can be  
obtained via formation of an interaction bond of the formula  $-(\text{CH}_2)_n-\text{S}-\text{CH}_2-\text{C}-$ ,  
wherein  $n = 1$  or  $2$ , which is possible, for example, through incorporation

of Cys or homoCys and reaction of its free SH group with, e.g., bromoacetylated Lys, Orn, Dab or Dap.

Peptide bonds (-CO-NH-) within the peptide may be substituted by N-methylated bonds (-N(CH<sub>3</sub>)-CO-), ester bonds (-C(R)H-C-O-O-C(R)-N-),  
 5 ketomethylen bonds (-CO-CH<sub>2</sub>-),  $\alpha$ -aza bonds (-NH-N(R)-CO-), wherein R is any alkyl, e.g., methyl, carba bonds (-CH<sub>2</sub>-NH-), hydroxyethylene bonds (-CH(OH)-CH<sub>2</sub>-), thioamide bonds (-CS-NH-), olefinic double bonds (-CH=CH-), retro amide bonds (-NH-CO-), peptide derivatives (-N(R)-CH<sub>2</sub>-CO-), wherein R is the "normal" side chain, naturally presented on  
 10 the carbon atom.

These modifications can occur at any of the bonds along the peptide chain and even at several (2-3) at the same time. Preferably, but not in all cases necessary, these modifications should exclude anchor amino acids.

Natural aromatic amino acids, Trp, Tyr and Phe, may be substituted for  
 15 synthetic non-natural acid such as TIC, naphthylelanine (Nol), ring-methylated derivatives of Phe, halogenated derivatives of Phe or o-methyl-Tyr.

***Tumor HLA-restricted antigens:***

The references recited in the following Table provide examples of human MHC class I, tumor HLA-restricted peptides derived from tumor  
 20 associated antigens (TAA) or protein markers associated with various cancers.

Cancer	TAA/Marker	HLA	Reference
Transitional cell carcinoma	Uroplakin II	HLA-A2	WO 00/06723
Transitional cell carcinoma	Uroplakin Ia	HLA-A2	WO 00/06723
Carcinoma of the prostate	prostate specific antigen	HLA-A2	WO 00/06723
Carcinoma of the prostate	prostate specific membrane antigen	HLA-A2	WO 00/06723



Carcinoma of the prostate	prostate acid phosphatase	HLA-A2	WO 00/06723
Breast cancer	BA-46	HLA-A2	WO 00/06723
Breast cancer	Muc-1	HLA-A2	WO 00/06723
Melanoma	Gp100	HLA-A2	Reference 54
Melanoma	MART1	HLA-A2	Reference 54
All tumors	Telomerase	HLA-A2	Reference 54
Leukemia	TAX	HLA-A2	Reference 54
Carcinomas	NY-ESO	HLA-A2	Reference 54
Melanoma	MAGE-A1	HLA-A2	Reference 54
Melanoma	MAGE-A3	HLA-A24	Reference 54
Carcinomas	HER2	HLA-A2	Reference 54
Melanoma	Beta-catenine	HLA-A24	Reference 54
Melanoma	Tyrosinase	HLA-DRB1	Reference 54
Leukemia	Bcr-abl	HLA-A2	Reference 54
Head and neck	Caspase 8	HLA-B35	Reference 54

***Viral HLA-restricted antigens:***

The references recited in the following Table provide examples of human MHC class I, viral HLA-restricted peptides derived from viral antigens associated with various cancers.

Disease	Viral antigen	HLA
AIDS (HTLV-1)	HIV-1 RT 476-484	HLA-A2
Influenza	G I L G F V F T L (SEQ ID NO:10)	HLA-A2
CMV disease	CMV	HLA-A2
Burkitts Lymphoma	TAX	HLA-A2

Hepatitis C	HCV	HLA-A2
Hepatitis B	HBV pre-S protein 85-66 S T N R Q S G R Q (SEQ ID NO:11)	HLA-A2
HTLV-1 Leukemia	HTLV-1 tax 11-19	HLA-A2
Hepatitis	HBV surface antigen 185-194	HLA-A2

***Soluble MHC class I molecules:***

Recombinant MHC class I and class II complexes which are soluble and which can be produced in large quantities are described in, for example, references 23, 24 and 41-53 and further in U.S. Patent Application No. 09/534,966 and PCT/IL01/00260 (published as WO 01/72768).

Soluble MHC class I molecules are available or can be produced for any of the MHC haplotypes, such as, for example, HLA-A2, HLA-A1, HLA-A3, HLA-A24, HLA-A28, HLA-A31, HLA-A33, HLA-A34, HLA-B7, HLA-B45 and HLA-Cw8, following, for example the teachings of PCT/IL01/00260, as their sequences are known and can be found at the kabat data base.

Such soluble MHC class I molecules can be loaded with suitable HLA-restricted antigens and used for vaccination of Non-human mammal having cells expressing the human major histocompatibility complex (MHC) class I as is further detailed hereinbelow.



***Non-human mammal having cells expressing the human major histocompatibility complex (MHC) class I:***

Non-human mammal having cells expressing a human major histocompatibility complex (MHC) class I and devoid of self major histocompatibility complex (MHC) class I can be produced using (i) the sequence information provided in the kabbat data base, and pertaining to human MHC haplotypes, such as, for example, HLA-A2, HLA-A1, HLA-A3, HLA-A24, HLA-A28, HLA-A31, HLA-A33, HLA-A34, HLA-B7, HLA-B45 and HLA-Cw8, (ii) conventional constructs preparation techniques, as described in, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); and (iii) conventional gene knock-in/knock-out techniques as set forth, for example, in United States Patents 5,487,992, 5,464,764, 5,387,742, 5,360,735, 5,347,075, 5,298,422, 5,288,846, 5,221,778, 5,175,385, 5,175,384, 5,175,383, 4,736,866; in International Publications WO 94/23049, WO93/14200, WO 94/06908 and WO 94/28123; as well as in Burke and Olson, Methods in Enzymology, 194:251-270, 1991; Capecchi, Science 244:1288-1292, 1989; Davies *et al.*, Nucleic Acids Research, 20 (11) 2693-2698, 1992; Dickinson *et al.*, Human Molecular Genetics, 2(8): 1299-1302, 1993; Duff and Lincoln, "Insertion of a pathogenic mutation into a yeast artificial chromosome containing the human APP gene and expression in ES cells", Research Advances in Alzheimer's Disease and Related Disorders, 1995; Huxley *et al.*, Genomics, 9:742-750 1991; Jakobovits *et al.*, Nature, 362:255-261, 1993; Lamb *et al.*, Nature Genetics, 5:

22-29, 1993; Pearson and Choi, Proc. Natl. Acad. Sci. USA, 1993. 90:10578-82; Rothstein, Methods in Enzymology, 194:281-301, 1991; Schedl *et al.*, Nature, 362: 258-261, 1993; Strauss *et al.*, Science, 259:1904-1907, 1993.

- 5 Of particular interest is the paper by Pascolo *et al.*, published in J. Exp. Med. 185: 2043-2051, 1997, which describe the preparation of mice expressing the human HLA-A2.1, H-2Db and HHD MHC class I molecules and devoid of mice MHC class I altogether.

***Identifiable moieties:***

- 10 In some aspects thereof, the present invention employ conjugates of an antibody and an identifiable moiety. To this end, in one example, first and second polynucleotides encoding the antibody and the identifiable moiety, respectively, are ligated in frame, so as to encode an immunolabel. The following table provide examples of sequences of identifiable moieties.

Identifiable Moiety	Amino Acid sequence (Genebank Accession No.)	Nucleic Acid sequence (Genebank Accession No.)
Green Fluorescent protein	AAL33912	AF435427
Alkaline phosphatase	AAK73766	AY042185
Peroxidase	NP_568674	NM_124071
Histidine tag	AAK09208	AF329457
Myc tag	AF329457	AF329457
Biotin lygase tag	NP_561589	NC_003366
orange fluorescent protein	AAL33917	AF435432
Beta galactosidase	NM_125776	NM_125776
Fluorescein isothiocyanate	AAF22695	AF098239
Streptavidin	S11540	S11540

15

***Therapeutic moieties:***

- In some aspects thereof, the present invention employ conjugates of an antibody and a therapeutic moiety. To this end, in one example, first and second polynucleotides encoding the antibody and the therapeutic moiety, respectively, are ligated in frame, so as to encode an immunotoxin. The following table provide examples of sequences of therapeutic moieties.
- 20



Therapeutic Moiety	Amino Acid sequence (Genebank Accession No.)	Nucleic Acid sequence (Genebank Accession No.)
Pseudomonas exotoxin	AAB25018	S53109
Diphtheria toxin	E00489	E00489
interleukin 2	CAA00227	A02159
CD3	P07766	X03884
CD16	AAK54251	AF372455
interleukin 4	P20096	ICRT4
HLA-A2	P01892	K02883
interleukin 10	P22301	M57627
Ricin A toxin	225988	A23903

***Chemical conjugates:***

Many methods are known in the art to conjugate or fuse (couple) molecules of different types, including peptides. These methods can be used according to the present invention to couple an antibody another moiety, such as a therapeutic moiety or an identifiable moiety, to thereby provide an immunotoxin or immunolabel.

Two isolated peptides can be conjugated or fused using any conjugation method known to one skilled in the art. A peptide can be conjugated to an antibody of interest, using a 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester (also called N-succinimidyl 3-(2pyridyldithio)propionate) ("SDPD") (Sigma, Cat. No. P-3415), a glutaraldehyde conjugation procedure or a carbodiimide conjugation procedure.

***SPDP conjugation:***

Any SPDP conjugation method known to those skilled in the art can be used. For example, in one illustrative embodiment, a modification of the method of Cumber et al. (1985, Methods of Enzymology 112: 207-224) as described below, is used.

A peptide, such as an identifiable or therapeutic moiety, (1.7 mg/ml) is mixed with a 10-fold excess of SPDP (50 mM in ethanol) and the antibody is mixed with a 25-fold excess of SPDP in 20 mM sodium phosphate, 0.10 M

NaCl pH 7.2 and each of the reactions incubated, e.g., for 3 hours at room temperature. The reactions are then dialyzed against PBS.

The peptide is reduced, e.g., with 50 mM DTT for 1 hour at room temperature. The reduced peptide is desalted by equilibration on G-25 column (up to 5 % sample/column volume) with 50 mM  $\text{KH}_2\text{PO}_4$  pH 6.5. The reduced peptide is combined with the SPDP-antibody in a molar ratio of 1:10 antibody:peptide and incubated at 4 °C overnight to form a peptide-antibody conjugate.

***Glutaraldehyde conjugation:***

Conjugation of a peptide (e.g., an identifiable or therapeutic moiety) with an antibody can be accomplished by methods known to those skilled in the art using glutaraldehyde. For example, in one illustrative embodiment, the method of conjugation by G.T. Hermanson (1996, "Antibody Modification and Conjugation, in Bioconjugate Techniques, Academic Press, San Diego) described below, is used.

The antibody and the peptide (1.1 mg/ml) are mixed at a 10-fold excess with 0.05 % glutaraldehyde in 0.1 M phosphate, 0.15 M NaCl pH 6.8, and allowed to react for 2 hours at room temperature. 0.01 M lysine can be added to block excess sites. After-the reaction, the excess glutaraldehyde is removed using a G-25 column equilibrated with PBS (10 % v/v sample/column volumes)

***Carbodiimide conjugation:***

Conjugation of a peptide with an antibody can be accomplished by methods known to those skilled in the art using a dehydrating agent such as a carbodiimide. Most preferably the carbodiimide is used in the presence of 4-dimethyl aminopyridine. As is well known to those skilled in the art, carbodiimide conjugation can be used to form a covalent bond between a carboxyl group of peptide and an hydroxyl group of an antibody (resulting in the formation of an ester bond), or an amino group of an antibody (resulting in the formation of an amide bond) or a sulfhydryl group of an antibody (resulting in the formation of a thioester bond).



Likewise, carbodiimide coupling can be used to form analogous covalent bonds between a carbon group of an antibody and an hydroxyl, amino or sulfhydryl group of the peptide. See, generally, J. March, *Advanced Organic Chemistry: Reaction's, Mechanism, and Structure*, pp. 349-50 & 372-74 (3d ed.), 1985. By means of illustration, and not limitation, the peptide is conjugated to an antibody via a covalent bond using a carbodiimide, such as dicyclohexylcarbodiimide. See generally, the methods of conjugation by B. Neises et al. (1978, *Angew Chem., Int. Ed. Engl.* 17:522; A. Hassner et al. (1978, *Tetrahedron Lett.* 4475); E.P. Boden et al. (1986, *J. Org. Chem.* 50:2394) and L.J. Mathias (1979, *Synthesis* 561).

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

### EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual

Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996).

Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader.



## **MATERIALS AND EXPERIMENTAL METHODS**

### ***Production of Biotinylated scMHC/peptide Complexes:***

Single-chain MHC/peptide complexes were produced by *in vitro* refolding of inclusion bodies produced in *E. coli*, as described previously (23, 24, U.S. Patent Application No. 09/534,966 and PCT/IL01/00260 (published as WO 01/72768). Biotinylation was performed using the BirA enzyme (Avidity, Denver, CO) as previously described (25).

### ***Mice immunization:***

D<sup>b</sup>/X β2 microglobulin (β2m) null mice, transgenic for a recombinant HLA-A2.1/D<sup>b</sup>-β2 microglobulin single chain (HHD mice) (26) were immunized with an emulsion containing purified protein-derived peptide of tuberculin (PPD) covalently coupled with HLA-A2/G9-209 complexes, as described previously (17). Briefly, mice were initially immunized subdermally and subsequently subcutaneously for two-week intervals for a period of 3-5 months with 20-30 μg/mice of the antigenic mixture in incomplete Freund's adjuvant. Spleens were collected two weeks after the last immunization.

### ***Library construction and selection of phage-antibodies on biotinylated complexes:***

Total RNA was isolated from immunized mice and an antibody scFv library was constructed by room temperature-PCR from the mRNA as described (27). The scFv repertoire was cloned as an *SfiI-NotI* fragment into the pCANTAB5E or pCC-CBD phagemid vectors (28). The complexity of both libraries was 1 x 10<sup>8</sup> independent clones. For panning, biotinylated scHLA-A2/G9-209M complexes (20 μg) were incubated with streptavidin-coated magnetic beads (2 x 10<sup>8</sup>), washed, and incubated with 10<sup>11</sup> cfu of the libraries (1 hour at room temperature). Starting with the 2<sup>nd</sup> round, panning was performed in the presence of an excess (5 μg) of scHLA-A2/G9-280V complexes. Beads were washed extensively 10-12 times with 2 % MPBS + 0.1 % TWEEN20. Bound phages were eluted by using 1 ml of Triethylamine (100 mM, pH 12) for 5 minutes at room temperature followed

by neutralization with 0.1 ml of 1 M Tris-HCl, pH 7.4. Eluted phages were expanded in exponentially growing *E. coli* TG1 cells that were later superinfected with M13KO7 helper phage as described (28).

***Expression and purification of soluble recombinant scFv and scFv-P38 fusion protein:***

The G1 scFv gene was rescued from the phage clone by PCR and was subcloned into the phagemid vector pCANTAB6 by using the *SfiI-NotI* cloning sites. A Myc and hexahistidine tags were fused to the C-terminus of the scFv gene. The scFv antibody was expressed in BL21  $\lambda$ DE3 cells as previously described (29) and purified from the periplasmic fraction by metal-ion affinity chromatography. For the expression of the G1scFv-PE38 fusion protein, the scFv gene was subcloned as an *NcoI-NotI* fragment into the plasmid pIB-NN, which encodes the translocation and ADP-ribosylation domains of PE (PE38). Expression in BL21  $\lambda$ DE3 cells, refolding from inclusion bodies, and purification of G1scFv-PE38 was performed as previously described (30).

***ELISA with phage clones and purified scFv or scFv-PE38:***

Binding specificity studies were performed by ELISA using biotinylated scMHC-peptide complexes. Briefly, ELISA plates (Falcon) were coated overnight with BSA-biotin (1  $\mu$ g/well), washed, incubated (1 hour, room temperature) with streptavidin (1  $\mu$ g/well), again washed extensively and further incubated (1 hour, room temperature) with 0.5  $\mu$ g of MHC/peptide complexes. Plates were blocked with PBS/2 % milk (30 minutes, room temperature), incubated with phage clones (about  $10^9$  phages/well, 1 hour, room temperature) or 0.5-1  $\mu$ g of soluble scFv or scFv-PE38, and afterwards washed with 1:1000 HRP-conjugated/anti-M13, anti-*myc* antibody or anti-PE antibody, respectively. The HLA-A2-restricted peptides used for specificity studies are gp100 (154): KTWGQYWQV (SEQ ID NO:1); gp100 (209): IMDQVPFSV (SEQ ID NO:2); gp100 (280): YLEPGPVTV (SEQ ID NO:3); MUC1: LLLTVLTVL (SEQ ID NO:4); HTLV-1 (TAX): LLFGYPVYV (SEQ ID



NO:5); hTEroom temperature (540): ILAKFLHWL (SEQ ID NO:6); hTEroom temperature (865): RLVDDFLLV (SEQ ID NO:7).

***Flow Cytometry:***

The B cell line RMAS-HHD transfected with a single-chain  
5  $\beta$ 2M-HLA-A2 gene (26) or the EBV-transformed B-lymphoblast JY cells ( $10^6$   
cells) were washed twice with serum-free RPMI and incubated overnight at 26  
 $^{\circ}\text{C}$  or 37  $^{\circ}\text{C}$ , respectively, in medium containing 100  $\mu\text{M}$  of the peptide. The  
APCs were subsequently incubated at 37  $^{\circ}\text{C}$  for 2-3 hours to stabilize cell  
surface expression of MHC-peptide complexes, followed by incubation with  
10 recombinant scFv (10-50  $\mu\text{g}/\text{ml}$ , 60-90 minutes, 4  $^{\circ}\text{C}$ ) in 100  $\mu\text{l}$ . The cells were  
then washed, incubated with FITC-labeled anti-Myc antibody (30-45 minutes, 4  
 $^{\circ}\text{C}$ ), and finally washed and analyzed by a FACStar flow cytometer (Becton  
Dickinson).

***Competition binding assays:***

15 Flexible ELISA plates were coated with BSA-biotin and scMHC-peptide  
complexes (10  $\mu\text{g}$  in 100  $\mu\text{l}$ ) were immobilized thereto. The recombinant  
G1scFv-PE38 was labeled with [ $^{125}\text{I}$ ] using the Bolton-Hunter reagent. Labeled  
protein was added to wells as a tracer ( $3\text{-}5 \times 10^5$  CPM/well) in the presence of  
increasing concentrations of the cold G1scFv-PE38 as a competitor and  
20 incubated at room temperature for 1 hour in PBS. The plates were washed  
thoroughly with PBS and the bound radioactivity was determined by a gamma  
counter. The apparent affinity of the G1scFv-PE38 was determined by  
extrapolating the concentration of a competitor necessary to achieve 50%  
inhibition of [ $^{125}\text{I}$ ]-labeled G1scFv-PE38 binding to the immobilized  
25 scMHC-peptide complex. Non-specific binding was determined by adding a  
20-40-fold excess of unlabeled Fab.

***Cytotoxicity assays:***

RMAS-HHD and JY APCs were loaded with the G9-209 peptide and  
control peptides as previously described. Peptide-loaded cells were  
30 subsequently incubated with increasing concentrations of G1scFv-PE38 and the

inhibition of protein synthesis was determined by measuring the uptake of <sup>3</sup>H-Leucine into cellular proteins, as previously described (30). IC<sub>50</sub> was determined as the concentration of G1scFv-PE38 required to inhibit protein synthesis by 50 %. In competition assays, excesses of specific and non-specific HLA-A2-peptide complexes (35-50 µg/well) were added to wells 15 minutes before the addition of G1scFv-PE38.

### **EXPERIMENTAL RESULTS**

#### ***Generation of recombinant single-chain MHC-peptide complexes with the melanoma gp100-derived peptide G9-209M:***

Gp100 is a melanocyte lineage-specific membrane glycoprotein consisting of 661 amino acids that is expressed in most melanoma cells (19-22). This protein is recognized by many HLA-A2-restricted, melanoma-reactive, tumor infiltrating-lymphocytes (TILs) that have been isolated from melanoma patients (19-22). Several T cell HLA-A2-restricted epitopes have been identified in gp100; they have been improved in MHC anchor positions for enhanced immunogenicity without altering T-cell specificity (31). The G9-209M (IMDQVPFSV, SEQ ID NO:2) peptide is one of a three major immunogenic epitopes (19-22). Recombinant MHC-peptide complexes that present the G9-209M peptide were generated by using a single-chain MHC (scMHC) construct expressed in *E. coli* that has been described previously (23, 24, U.S. Patent Application No. 09/534,966 and PCT/IL01/00260 (published as WO 01/72768). The scMHC-peptide complexes are produced by *in vitro* refolding of inclusion bodies in the presence of the G9-209M or other HLA-A2-restricted peptides, followed by a purification protocol employing ion-exchange chromatography. The refolded gp100-derived and control scHLA-A2-peptide complexes were very pure, homogenous, and monomeric, as determined by analysis on SDS-PAGE and gel filtration chromatography. The G9-209M-containing scHLA-A2 complexes have been previously shown to



be functional, by their ability to stimulate specific CTL lines and clones and stain G9-209M-specific T cells in the form of tetramers (23, 24).

***Construction of an antibody scFv phage library and selection of a phage that binds HLA-A2/G9-209M complexes with TCR-like specificity:***

5 For immunization purposes PPD was coupled to the purified complex and the D<sup>b</sup>-/ X β2 microglobulin (β2m) null mice transgenic for a recombinant HLA-A2.1/D<sup>b</sup>-β2 microglobulin single chain (HHD mice) (26) was immunized therewith. These mice combine classical HLA transgenesis with selective  
10 destruction of murine class I H-2. Hence, unlike the classical HLA transgenics, these mice showed only HLA-A2.1-restricted responses with multi-epitope proteins such as intact viruses. Moreover, it is presume that these mice are a useful tool for immunization with HLA-A2-peptide complexes because they should be largely tolerant to HLA-A2 as a B-cell immunogen and thus may favor the generation of an antibody response directed against the  
15 MHC-restricted epitope when in complex with HLA-A2 (the specific tumor-associated peptide). PPD was used for conjugation because it is a highly reactive T cell immunogen (17).

Total spleen mRNA was isolated from immunized mice and reverse transcribed to cDNA. Specific sets of degenerated primers were used to  
20 PCR-amplify the cDNA segments corresponding to the immunoglobulin heavy and light chain variable domains (27). The VH and VL PCR pools were assembled into a scFv repertoire by a PCR overlap extension reaction and subsequently cloned into the pCANTAB5E phagemid vector or to the phagemid vector pCC-Gal6(Fv) in which the scFv is expressed as an in frame fusion  
25 protein with a cellulose-binding domain (CBD) (28). The resulting libraries were transduced into *E. coli* TG1 cells by electroporation and expressed as fusion with the minor phage coat protein pIII after rescue with a helper phage. The library complexity consisted of  $1 \times 10^8$  independent clones using both types of vectors.

The library was subjected to 3-4 panning cycles followed by elution of bound phages and reamplification in *E. coli*. To enhance the efficiency of selection biotinylated scMHC-peptide complexes were used. A BirA sequence tag for site-specific biotinylation was engineered at the C-terminus of the HLA-A2 gene as previously described (25). Several selection strategies were employed, the most successful of which resulted in the isolation of specific binders consisting of panning protocols with a negative depletion step starting from the 2<sup>nd</sup> round of panning. The specific HLA-A2/G9-209M biotinylated complexes were immobilized onto streptavidin-coated magnetic beads and the library was incubated with the immobilized complex in the presence of a large excess of HLA-2 complexes that displayed a different gp100-derived epitope, the G9-280V peptide. When this strategy is used G9-209M-specific phage will bind to streptavidin-biotin-immobilized complexes that are captured by a magnetic force, whereas pan-MHC binders that are not specific to the G9-209M peptide in the complex will bind to the non-specific complex in the solution and thus can be separated and removed from the specific phage. As shown in Table 1 below, a progressive, marked enrichment for phages that bind the immobilized complexes was observed after 3-4 rounds of panning, two of which were performed with the negative depletion strategy.

**Table 1: Phage selection on scHLA-A2/G9-209M complexes**

Library	Cycle	Input	Output	Enrichment
scFv	1	$1 \times 10^{12}$	$1 \times 10^4$	-
	2	$5 \times 10^{11}$	$1 \times 10^5$	10
	3	$5 \times 10^{11}$	$1 \times 10^9$	10,000
scFv-CBD	1	$5 \times 10^9$	$1 \times 10^4$	-
	2	$5 \times 10^{11}$	$1 \times 10^5$	10
	3	$5 \times 10^{11}$	$1 \times 10^8$	1,000

A 4<sup>th</sup> round of selection resulted with similar enrichments as observed in round 3.



Polyclonal phage ELISA was performed to determine phage specificity on biotinylated recombinant scMHC-peptide complexes immobilized to BSA-Biotin-streptavidin-coated immunoplates. The BSA-biotin-streptavidin spacer enables the correct presentation of the complexes, which can be distorted by direct binding to plastic. Phage analyzed already after the 2<sup>nd</sup> and more dramatically, after the 3<sup>rd</sup> round of panning revealed a unique specificity pattern only directed toward the specific G9-209M-containing HLA-A2 complexes (Figures 1A-B). No binding was observed with control HLA-A2 complexes that display the gp100-derived epitope, G9-280V or the telomerase-derived epitope 654.

Individual monoclonal phage clones were isolated from the population of phages from the last round of panning (no further enrichment observed after a 4<sup>th</sup> round) and re-screened for specificity by phage ELISA (Figures 2A-B). Of the 93 clones tested, 85 (91 %) reacted with the HLA-A2/G9-209M complex (Figure 2A). Seventy-seven out of the 85 reactive clones (90 %) reacted specifically with the specific HLA-A2/G9-209M complex but not with the control G9-280V-containing complex (Figure 2B). Only a small percentage of the clones (5/93; 5 %) did not exhibit peptide specificity (Figure 2B). Thus, the panning procedure yielded a successful enrichment of phage antibodies with TCR-like specificity toward the HLA-A2/G9-209M complex. Fingerprint analysis by means of multicutter restriction enzyme digestion revealed that 50 positive, HLA-A2/G9-209M-specific clones had a similar digestion pattern, indicating that all are similar (data not shown). Similar results were obtained with the two libraries. Since they were constructed from the same genetic material (the same pool of mRNA), only phage clones derived from the pCANTAB5E scFv library were further characterized.

DNA sequencing of VH and VL variable domains from 10 clones revealed that all were identical (data not shown), suggesting that they were all derived from a single productive antibody VH/VL combinatorial event.

***Characterization of the soluble recombinant scFv antibody with TCR-like specificity:***

DNA sequencing revealed that the antibody VH sequence belongs to the mouse heavy chains subgroup III (D) and the VL sequence to mouse kappa light chains group IV (according to Kabat). The nucleotide sequence (SEQ ID NO:8) and deduced amino acid sequence (SEQ ID NO:9) are shown in Figure 3A. To further characterize the binding specificity and the biological properties of the selected scFv antibody, termed G1, two expression systems were used; for the first, the scFv was subcloned into the phagemid vector pCANTAB6 in which a myc and a hexahistidine tag is fused to the C-terminus of the scFv gene. The second was a T7 promoter-driven expression system in which the scFv gene is fused to a truncated form of *Pseudomonas* Exotoxin A (PE38) to generate a scFv-immunotoxin (12). This truncated form of PE contains the translocation and ADP-ribosylation domains of whole PE but lacks the cell-binding domain, which is replaced by the scFv fragment fused at the N-terminus of the truncated toxin. The G1 scFv was produced in *E. coli* BL21 ( $\lambda$ DE3) cells by secretion and was purified from periplasmic fractions by metal affinity chromatography using the hexahistidine tag fused to the C-terminus (Figure 3B). The G1 scFv-PE38 was expressed in BL21 cells and upon induction with IPTG, large amounts of recombinant protein accumulated as intracellular inclusion bodies. SDS-PAGE showed that inclusion bodies from cultures expressing G1 scFv-PE38 contained more than 90 % recombinant protein. Using established renaturation protocols, G1 scFv-PE38 was refolded from solubilized inclusion bodies in a redox-shuffling refolding buffer and was thereafter purified by ion-exchange chromatography on Q-Sepharose and MonoQ columns, and later by size-exclusion chromatography. A highly purified G1 scFv-PE38 fusion protein with the expected size of 63 kDa was obtained as analyzed by SDS-PAGE under non reducing conditions (Figure 3C). The molecular profile of the G1scFv and G1scFv-immunotoxin was analyzed by size-exclusion chromatography and revealed a single protein peak



in a monomeric form with an expected molecular mass of 26 and 63 kDa, respectively (data not shown). The yield of the refolded G1 scFv-immunotoxin was about 2 %, thus, 2 mg of highly pure protein could be routinely obtained from the refolding of 100 mg of protein derived from inclusion bodies containing 80-90 % of recombinant protein. This yield is similar to previously reported scFv-immunotoxins that expressed well and were produced using a similar expression and refolding system (30). The yield of the G1scFv was 3 mg from a 1-liter bacterial culture.

The binding specificity of the soluble purified G1 scFv antibody and G1 scFv-PE38 fusion protein was determined by ELISA assays on biotinylated MHC-peptide complexes immobilized to wells through BSA-biotin-streptavidin to ensure correct folding of the complexes, which can be distorted by direct binding to plastic. The correct folding of the bound complexes and their stability during the binding assays were determined by their ability to react with the conformational, specific monoclonal antibody w6/32, which binds HLA complexes only when folded correctly and when it contains peptide (data not shown). When we used the soluble-purified G1 scFv or G1scFv-PE38 protein, the ELISA assays revealed a very specific recognition pattern corresponding to the hallmarks of MHC-restricted T-cell specificity (Figure 4). The G1 scFv selected to bind the G9-209M-containing HLA-A2 complex reacted only with the specific complex and not with complexes displaying the G9-280 and G9-154 gp100-derived MHC-peptide complexes nor to other control complexes containing HLA-A2-restricted telomerase-derived epitopes 540 and 865 (32), a MUC1-derived peptide (33), or the HTLV-1-derived TAX peptide (34) (Figure 4). In these assays the binding was detected with an anti-PE38 antibody. Similar results were obtained when using the unfused G1 scFv antibody where detection was performed with anti-Myc tag antibody (data not shown). Thus, this antigen-specific scFv fragment exhibits binding characteristics and the fine specificity of a TCR-like molecule. The G1 scFv or G1 scFv-PE38 did not recognize the peptide alone nor empty HLA-A2 molecules (which are difficult

to produce because they are unstable in the absence of a peptide), neither streptavidin nor other protein antigens (data not shown).

Next, the binding properties of the TCR-like soluble purified G1 scFv-PE38 were determined using a saturation ELISA assay in which  
5 biotinylated complexes were bound to BSA-biotin-streptavidine-coated plates to which increasing amounts of G1 scFv-PE38 were added. The binding of G1scFv-PE38 to the specific gp100-derived HLA-A2/G9-209M complex was dose-dependent and saturable (Figure 5A). Extrapolating the 50 % binding  
10 signal revealed that this antibody possessed high affinity, with a binding affinity in the nanomolar range. To determine the apparent binding affinity of the TCR-like scFv fragments to its cognate MHC-peptide complex, a competition binding assay was performed in which the binding of <sup>125</sup>I-labeled G1scFv-PE38 was competed with increasing concentrations of unlabeled protein. These  
15 binding assays revealed an apparent binding affinity in the low nanomolar range of 5 nM (Figure 5B). Importantly, these results underscore a previous success in isolating a high affinity scFv antibody with TCR-like specificity from the phage-displayed antibody repertoire of immunized HLA-A2 transgenic mice.

***Binding of G1 scFv to APCs displaying the gp100-derived epitope:***

To demonstrate that the isolated soluble G1 scFv can bind the specific  
20 MHC-peptide complex, not only in its recombinant soluble form but also in the native form, as expressed on the cell surface, two APC systems were utilized. One consisted of the murine TAP2-deficient RMA-S cells that were transfected with the human HLA-A2 gene in a single-chain format (26) (HLA-A2.1/Db-β2m single chain) (RMA-S-HHD cells). The gp100-derived  
25 peptide and control peptides were loaded on the RMA-S-HHD cells and the ability of the selected G1 scFv antibody to bind to peptide-loaded cells was monitored by FACS (Figures 6A-C). Peptide-induced MHC stabilization of the TAP2 mutant RMA-S-HHD cells was determined by analyzing the reactivity of the conformational anti HLA antibody w6/32 and the anti-HLA-A2 MAb  
30 BB7.2 with peptide loaded and unloaded cells (Figures 6A and B). The G1



scFv, which recognized the G9-209M-containing HLA-A2 complex, reacted only with RMA-S-HHD cells loaded with the G9-209M peptide but not with cells loaded with the G9-280 peptide (Figure 6C) or control cells not loaded with peptide. The G1 scFv did not bind to cells loaded with other HLA-A2-restricted control peptides such as TAX, MUC1 or telomerase-derived peptides used for the specificity analysis (see Figure 4).

A second type of APCs was also used, namely the EBV-transformed B lymphoblast JY cells, which express HLA-A2; these cells were incubated with the gp100-derived or control peptides. They are TAP+, and consequently, the displaying of the exogenously supplied peptide is facilitated by peptide exchange. Using this strategy, a similar binding specificity with the G1 scFv antibody was observed (data not shown). These results demonstrate that the scFv antibody can specifically recognize its corresponding native HLA-A2 complex *in situ* on the surface of cells.

#### 15 ***Cytotoxic activity of G1scFv-PE38 toward APCs:***

To determine the ability of the G1 scFv antibody to serve as a targeting moiety for T cell-like specific elimination of antigen-presenting cells, a G1scFv-PE38 molecule was constructed in which the very potent truncated form of *Pseudomonas* exotoxin A is fused to the C-terminus of the scFv gene and its ability to kill peptide-loaded APCs was tested. RMA-S-HHD or JY cells were loaded with the gp100-derived epitopes G9-209M and G9-280V as well as with other control HLA-A2-restricted peptides. FACS analysis with anti-HLA-A2 antibody revealed a similar expression pattern of HLA-A2 molecules with G9-209M, G9-280V, and other control peptide-loaded cells (Figure 6B). As shown in Figure 7A, cytotoxicity by G1scFv-PE38 was observed only on RMA-S-HHD cells loaded with the G9-209 peptide with an  $IC_{50}$  of 10-20 ng/ml. No cytotoxic activity was observed on RMA-S-HHD cells that were loaded with the gp100-derived G9-280V epitope or with other control HLA-A2-restricted peptides or cells that were not loaded with peptide. G9-209M-loaded RMA-S-HHD cells were not killed with an irrelevant

immunotoxin in which an anti human Lewis Y scFv antibody is fused to PE38 [B3(Fv)-PE38] (Figure 7A). In the EBV-transformed JY cells, which express normal TAP, the display of the exogenously-supplied peptide is facilitated by peptide exchange. Using this strategy, similar specific activity was observed in which G1scFv-PE38 kills only cells loaded with the G9-209M peptide (Figure 7B). Additional proof for specificity was demonstrated in competition experiments in which excess specific and control soluble scHLA-A2-peptide complex was present in solution, in order to compete for binding and inhibit cytotoxicity by G1 scFv-PE38. An example of this type of assay is shown in Figure 7C, in which excess soluble G9-209M-containing HLA-A2 but not the G9-280V/HLA-A2 complex competed and inhibited the cytotoxic activity of G1 scFv-PE38 toward G9-209M-loaded JY cells. These results further demonstrate the fine and unique specificity of the G1scFv antibody and its ability to serve as a targeting moiety to deliver a cytotoxic effector molecule with antigen (peptide)-specific, MHC-restricted specificity of T cells directed toward a human tumor T-cell epitope.

### ***DISCUSSION OF THE RESULTS***

In this example, the ability to select from an immune repertoire of murine scFv fragments a high affinity antibody (referred to herein as G1scFv) directed toward a human T-cell epitope derived from a cancer antigen, the melanoma-associated antigen gp100, was demonstrated.

G1scFv exhibits a very specific and special binding pattern; it can bind in a peptide-specific manner to HLA-A2 complexes. Hence, this is a recombinant antibody with T-cell antigen receptor-like specificity. In contrast to the inherent low affinity of TCRs, this molecule displays the high affinity binding characteristics of antibodies, while retaining TCR specificity.

This example strikingly demonstrates the power of the phage display approach and its ability to select especially fine specificities from a large repertoire of different antibodies.



The ability to select high-affinity TCR-like antibodies, despite the fact that such peptide-specific binders are thought to be rare and hence difficult to isolate, may result from the following considerations.

One is the mode of immunization and selection which included  
5 immunization of transgenic animal combined with the power of various selection strategies employed by phage display. It is believed that using HLA transgenic mice, such as HLA-A2 transgenic mice, is an advantage because they are usually tolerant to HLA complexes unless a new foreign peptide is presented on the complex. The ability to isolate TCR-like antibody molecules  
10 may represent a situation in which lymphocytes that were tolerant to HLA-A2 are now exposed to new epitopes contributed, in the example provided herein, by the melanoma gp100-derived peptide presented on HLA-A2. The panning procedure that combined an excess of non-specific complex in solution significantly contributed to the selection process and allowed to isolate a rare  
15 antibody clone (one out of  $10^8$ ).

Another important issue relates to the state of the antigen used in the selection process. The conformation of the antigen has to be as "natural" as possible, especially when produced in a recombinant form. As described in references 23 and 24 and in U.S. Patent Application No. 09/534,966 and  
20 PCT/IL01/00260 (published as WO 01/72768), it was found that *in vitro* refolding from inclusion bodies produced in *E. coli* of a single-chain MHC molecule complexed with various peptides yields large amounts of correctly folded and functional protein. The fact that the exemplary antibody G1scFv was isolated from a relatively small library of about  $10^8$  clones, yet is highly  
25 specific with an affinity in the nanomolar range, strongly indicates that the HLA-A2 transgenic mice that was used for immunization indeed developed high-affinity antibodies to the HLA-A2/G9-209 complexes. The observation that only a single anti-HLA-A2/G9-209 antibody was isolated may reflect that only one such specificity exists or that other specificities were not generated  
30 during the immune response because such a response could not be easily

generated and tolerated by the HLA-A2 transgenic mice. Quite astonishing is the fact that similar results were reported in the past for a murine MHC-peptide system where, using phage display, a recombinant TCR-like antibody directed toward a class I murine H-2K<sup>k</sup> molecule in complex with the influenza hemagglutinin peptide Ha<sub>255-262</sub> was isolated (17). Similar to the results presented here, of the 50 clones tested, seven reacted specifically with the H-2K<sup>k</sup>/Ha<sub>255-262</sub> complexes only, and not with other H-2K<sup>k</sup>/peptide complexes. Interestingly, the DNA sequences of these specific clones were determined and found to be identical (17). These anti-H-2K<sup>k</sup>/Ha<sub>255-262</sub> complexes antibodies, however, cannot be used to monitor antigen presentation and or kill antigen presenting cells of human origin.

Despite the fact that antibodies having a T-cell antigen receptor-like specificity are rare, the phage display approach can be applied to isolate recombinant antibodies with TCR-like specificity to a variety of MHC-peptide complexes related to various pathological conditions such as cancer, viral infections, and autoimmune diseases.

Recombinant antibodies with TCR-like specificity represent a new, valuable tool for future research in two major areas of tumor immunology. First, these antibodies may now be used to detect and directly visualize the presence of specific T-cell epitopes or MHC-peptide complexes by standard methods of flow cytometry and immuno-histochemistry. They should be very useful for the study and analysis of antigen presentation in cancer by determining the expression of specific tumor-related MHC-peptide complexes on the surface of tumor cells, metastasis, antigen-presenting cells, and lymphoid cells. Moreover, such antibodies can be used to analyze immunotherapy-based approaches by determining the alterations in MHC-peptide complex expression on antigen-presenting cells before, during, and after vaccination protocols with peptides or with APCs loaded with tumor cell extracts or dendritic-tumor cell hybrid vaccinations (7-11). Thus, questions relating to how and where certain events occur during antigen presentation may be directly addressed, for the first



time, and the expression of T-cell epitopes on the antigen-presenting cell may be visualized and quantitated.

Second, antibodies with such exquisitely fine specificity directed toward a very specific and unique human tumor antigen present new opportunities for use as targeting moieties for various antibody-based immunotherapeutic approaches. This includes using such antibodies to construct recombinant immunotoxins (12), fusion with cytokine molecules (37) or for bi-specific antibody therapy (38). The open question with respect to these applications relates to the low density of the specific epitope on the target cell's surface. It has been previously demonstrated, using the murine H-2K<sup>k</sup>/influenza hemagglutinin peptide complex and a similar antigen-presenting system that to achieve efficient killing with a TCR-like immunotoxin molecule, a density of several thousand particular MHC-peptide complexes are required for the selective elimination of APCs (39). The results of described herein support these findings, achieving a similar cytotoxic potential of a T-cell-like immunotoxin. To improve the targeting capabilities of these TCR-like antibody molecules, two antibody engineering approaches can be employed: (i) to increase the affinity of the parental antibody by affinity maturation strategies without altering its TCR-like fine specificity (40); and (ii) to increase the avidity of these recombinant monovalent molecules by making them bi-valent (38). Combining these strategies will result in second generation, improved molecules that will be valuable tools for immunotherapeutic approaches as well as serve as innovative research tools for studying the interaction of tumor cells and the human immune system.

25

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single

embodiment, may also be provided separately or in any suitable subcombination.

In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.



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## SEQUENCE LISTING

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 35 40 45  
 Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro Asp Ser Val  
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 Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr  
 65 70 75 80





## CLAIMS

1. An isolated antibody specifically binding with a binding affinity below 20 nanomolar to a human major histocompatibility complex (MHC) class I protein being complexed with a HLA-restricted antigen, and to a complex composed of a single chain human MHC and said HLA-restricted antigen, wherein said antibody does not bind said MHC class I protein in the absence of said HLA-restricted antigen, and wherein said antibody does not bind said HLA-restricted antigen in the absence of said human MHC class I protein and wherein said antibody binds with an antigen-specific, MHC-restricted specificity viral-infected cells or tumor cells naturally presenting said human MHC class I protein being complexed with said HLA-restricted antigen.

2. An isolated antibody specifically binding with a binding affinity below 20 nanomolar to a human major histocompatibility complex (MHC) class I protein being complexed with a HLA-restricted antigen, wherein said antibody does not bind said MHC class I protein in the absence of said HLA-restricted antigen, and wherein said antibody does not bind said HLA-restricted antigen in the absence of said human MHC class I protein, wherein said antibody binds with an antigen-specific MHC-restricted specificity viral-infected cells or tumor cells naturally presenting said human MHC class I protein being complexed with said HLA-restricted antigen, and wherein said antibody is obtained by screening an antibody library with a single chain human MHC complexed with said HLA-restricted antigen.

3. An isolated soluble antibody specifically binding with a binding affinity below 10 nanomolar to a soluble human major histocompatibility complex (MHC) class I protein complexed with a HLA-restricted tumor antigen, wherein said antibody does not bind said soluble human MHC class I protein in the absence of said HLA-restricted tumor antigen, wherein said antibody does not bind said HLA-restricted tumor antigen in the absence of said soluble human MHC class I protein, and wherein said soluble antibody binds with an antigen-specific MHC-restricted specificity tumor



cells naturally presenting human MHC class I protein complexed with said HLA-restricted tumor antigen.

4. The isolated antibody of claim 1 or 2, wherein said binding affinity is below 10 nanomolar.

5. The isolated antibody of claim 1 or 2, wherein said single chain human MHC is produced in bacteria.

6. A pharmaceutical composition comprising as the active ingredient the isolated antibody of claim 1, 2, 3, 4 or 5, and a pharmaceutically acceptable carrier.

7. A diagnostic composition for diagnosing of cancer or viral infection comprising the isolated antibody of claim 1, 2, 4 or 5 and an identifiable moiety.

8. A method of isolating an antibody specifically binding to a human major histocompatibility complex (MHC) class I protein complexed with a HLA-restricted antigen comprising:

(a) screening a plurality of antibodies with a bacterially produced complex of a single chain human MHC class I protein and a HLA-restricted antigen, wherein said single chain human MHC class I protein comprises a functional human  $\beta$ -2 microglobulin amino acid sequence covalently linked upstream to a functional human MHC class I heavy chain amino acid sequence; and

(b) selecting an antibody of said plurality of antibodies which specifically binds said bacterially produced complex,

thereby isolating the antibody specifically binding to the human MHC class I protein complexed with the HLA-restricted antigen.

9. Use of the isolated antibody of claim 1, 2, 4 or 5 for the manufacture of a medicament for treating melanoma or a viral infection wherein the virus is selected from the group consisting of CMV, HIV, Influenza, HBV and HCV.

10. A method of making an immunotoxin, the method comprising ligating a first polynucleotide encoding the isolated antibody of claim 1, 2, 3, 4 or 5, in frame with a second polynucleotide encoding a toxic moiety, so as to obtain a ligated polynucleotide and expressing said ligated polynucleotide in an expression system so as to obtain the immunotoxin.

11. A method of making an immunolabel, the method comprising ligating a first polynucleotide encoding the isolated antibody of claim 1, 2, 3, 4 or 5 in frame with a second polynucleotide encoding an identifiable moiety, so as to obtain a ligated polynucleotide and expressing said ligated polynucleotide in an expression system so as to obtain the immunolabel.

12. A method of detecting the presence and/or level of antigen presenting cells presenting an HLA-restricted antigen in a sample of cells, the method comprising:

interacting cells of the sample with the isolated antibody of claim 1, 2, 3, 4 or 5 or the diagnostic composition of claim 7; and

monitoring said interaction, thereby detecting the presence and/or level of the antigen presenting cells presenting the HLA-restricted antigen.

13. The isolated antibody of claim 1, 2, 3, 4 or 5, the pharmaceutical composition of claim 6 or the diagnostic composition of claim 7, wherein said MHC class I protein is selected from the group consisting of HLA-A2, HLA-A1, HLA-A3, HLA-A24, HLA-A28, HLA-A31, HLA-A33, HLA-A34, HLA-B7, HLA-B45 and HLA-Cw8.

14. The isolated antibody of claim 1, 2, 3, 4 or 5, the pharmaceutical composition of claim 6 or the diagnostic composition of claim 7, wherein the isolated antibody is conjugated to an identifiable moiety.



15. The isolated antibody of claim 14, the pharmaceutical composition of claim 14 or the diagnostic composition of claim 14, wherein said identifiable moiety is selected from the group consisting of a member of a binding pair and a label.

16. The isolated antibody, the pharmaceutical composition or the diagnostic composition of claim 15, wherein said member of said binding pair is an antigen.

17. The isolated antibody, the pharmaceutical composition, the diagnostic composition of claim 15, wherein said label is selected from the group consisting of a fluorescent protein and an enzyme.

18. The isolated antibody of claim 1, 2, 3, 4 or 5, the pharmaceutical composition of claim 6 or the diagnostic composition of claim 7, wherein the isolated antibody is conjugated to a therapeutic moiety.

19. The isolated antibody of claim 18, the pharmaceutical composition of claim 18 or the diagnostic composition of claim 18, wherein said therapeutic moiety is selected from the group consisting of a cytotoxic moiety, a toxic moiety, a cytokine moiety and a bi-specific antibody moiety.

20. The isolated antibody of claim 1, 2, 3, 4 or 5, the pharmaceutical composition of claim 6 or the diagnostic composition of claim 7, wherein said HLA-restricted antigen is a tumor HLA-restricted antigen.

21. The isolated antibody of claim 1, 2, 4 or 5, the pharmaceutical composition of claim 6 or the diagnostic composition of claim 7, wherein said HLA-restricted antigen is a viral HLA-restricted antigen.

22. The method of claim 8, wherein said plurality of antibodies are comprised in a phage display library which is produced by a method comprising:

immunizing a genetically engineered non-human mammal having cells expressing said human major histocompatibility complex (MHC) class I protein with a soluble form of a MHC class I protein being complexed with said HLA-restricted antigen;

isolating mRNA molecules from antibody producing cells of said non-human mammal;

thereby producing a phage display library displaying protein molecules encoded by said mRNA molecules.

23. The method of claim 22, wherein said non-human mammal is devoid of self MHC class I proteins.

24. The method of claim 22, wherein said soluble form of a MHC class I protein is a single chain MHC class I protein including a functional human  $\beta$ -2 microglobulin amino acid sequence covalently linked upstream to a functional human MHC class I heavy chain amino acid sequence.

25. An isolated nucleic acid comprising the polynucleotide as set forth in SEQ ID NO:8.

26. An isolated protein comprising the amino acid sequence as set forth in SEQ ID NO:9.

27. An isolated nucleic acid comprising a polynucleotide encoding the polypeptide as set forth in SEQ ID NO:9.

28. The antibody, the pharmaceutical composition or the diagnostic composition of claim 20, wherein said tumor HLA-restricted antigen is selected from the group consisting of Uroplakin II, Uroplakin Ia, prostate specific antigen, prostate specific membrane antigen, prostate acid phosphatase, BA-46, Muc-1, Gp100, MART1, Telomerase, TAX, NY-ESO, MAGE-A1, MAGE-A3, HER2, Beta-catenine, Bcr-abl, and Caspase 8.



29. The antibody, the pharmaceutical composition or the diagnostic composition of claim 20, wherein said tumor HLA-restricted antigen is Tyrosinase.

30. The antibody, the pharmaceutical composition or the diagnostic composition of claim 21, wherein said viral HLA-restricted antigen is selected from the group consisting of HIV-1 RT 476-484, GILGFVFTL (SEQ ID NO:10), CMV, TAX, HCV, HBV pre-S protein 85-66 STNRQSGRQ (SEQ ID NO:11), HTLV-1 tax 11-19 and HBV surface antigen 185-194.

31. The isolated antibody of any one of claims 1, 2, 3, 4, 5, 13, 14-17, 18-21, and 28-30, the pharmaceutical composition of any one of claims 6, 13, 14-17, 18-21, and 28-30 or the diagnostic composition of any one of claims 7, 13, 14-17, 18-21, and 28-30, wherein the antibody specifically binds with a binding affinity below 10 nanomolar to said human major histocompatibility complex (MHC) class I protein being complexed with said HLA-restricted antigen.

32. The use of claim 9, wherein said MHC class I protein is selected from the group consisting of HLA-A2, HLA-A1, HLA-A3, HLA-A24, HLA-A28, HLA-A31, HLA-A33, HLA-A34, HLA-B7, HLA-B45 and HLA-Cw8.

33. The method of claims 8, 11 or 12, wherein said MHC class I protein is selected from the group consisting of HLA-A2, HLA-A1, HLA-A3, HLA-A24, HLA-A28, HLA-A31, HLA-A33, HLA-A34, HLA-B7, HLA-B45 and HLA-Cw8.

34. The use of claim 9, wherein the isolated antibody is conjugated to an identifiable moiety.

35. The method of claim 8 or 12, wherein the isolated antibody is conjugated to an identifiable moiety.

36. The use of claim 9, wherein said HLA-restricted antigen is a tumor HLA-restricted antigen.

37. The method of claim 8, 11 or 12, wherein said HLA-restricted antigen is a tumor HLA-restricted antigen.

38. The use of claim 9, wherein the isolated antibody is conjugated to a therapeutic moiety.

39. The method of claim 8, 11 or 12, wherein the isolated antibody is conjugated to a therapeutic moiety.

40. A diagnostic composition for diagnosing of cancer comprising the isolated antibody of claim 3 and an identifiable moiety.

41. Use of the isolated antibody of claim 3 for the manufacture of a medicament for treating melanoma.



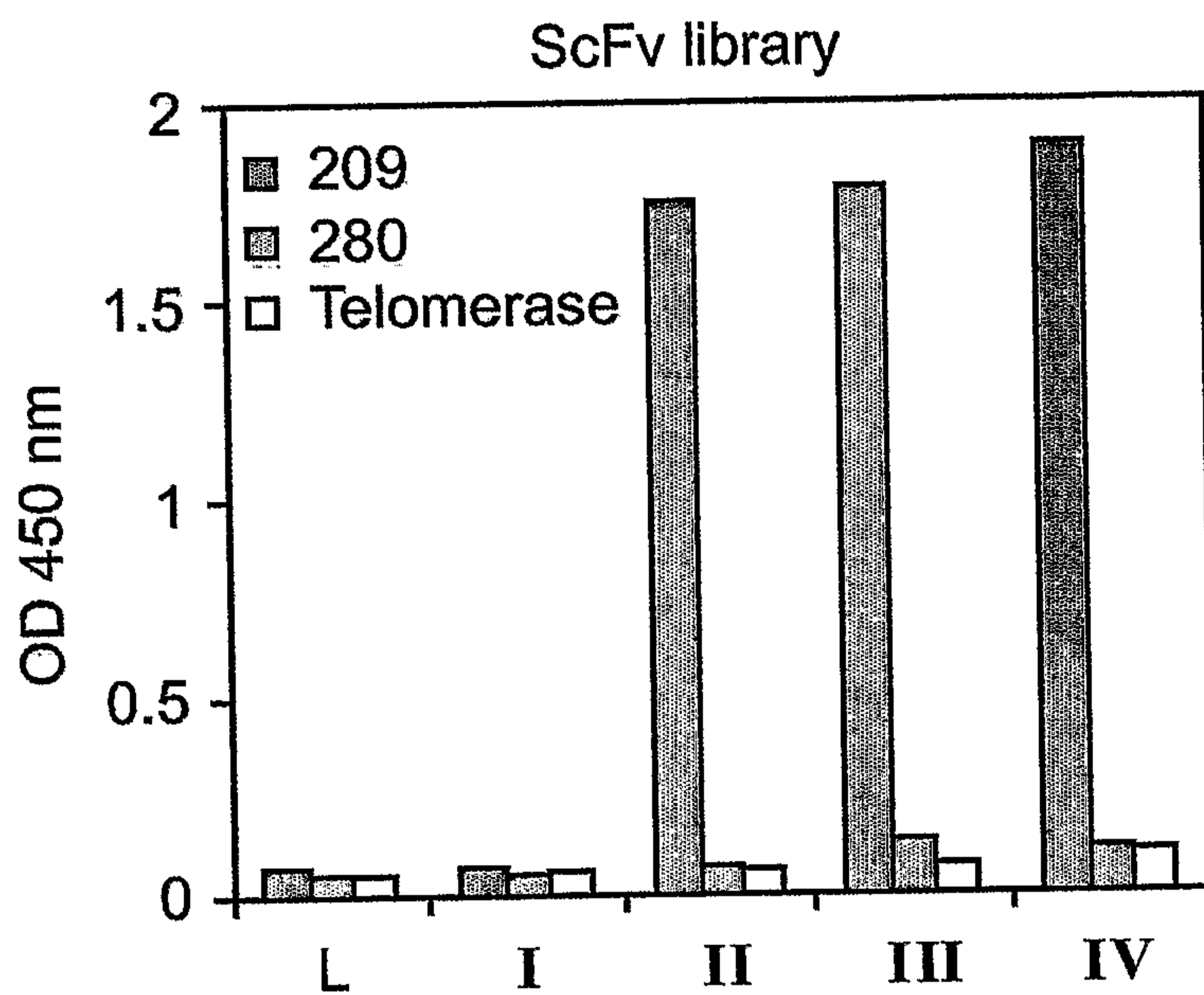


Fig. 1a

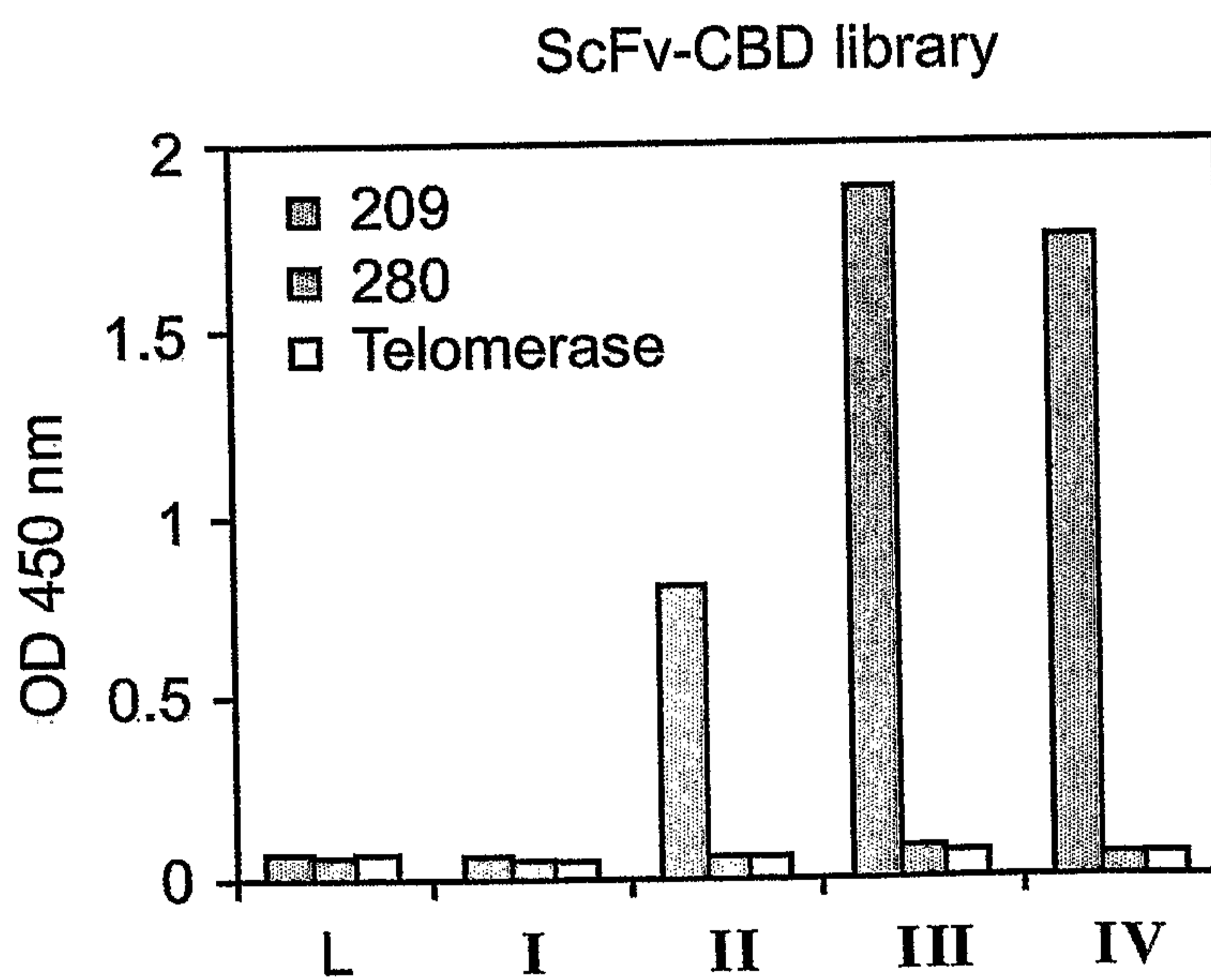


Fig. 1b

HLA-A2/G9-209

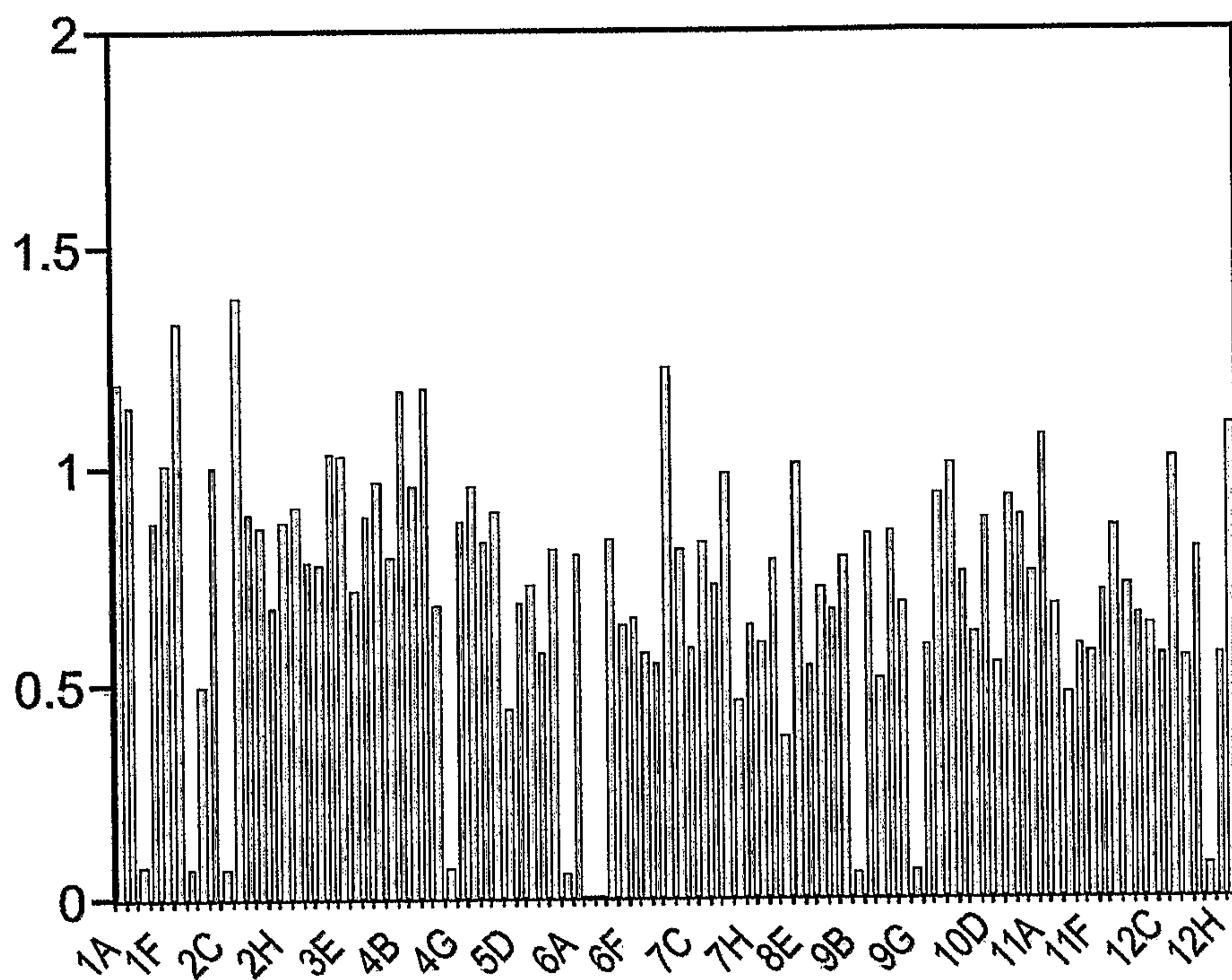


Fig. 2a

HLA-A2/G9-280

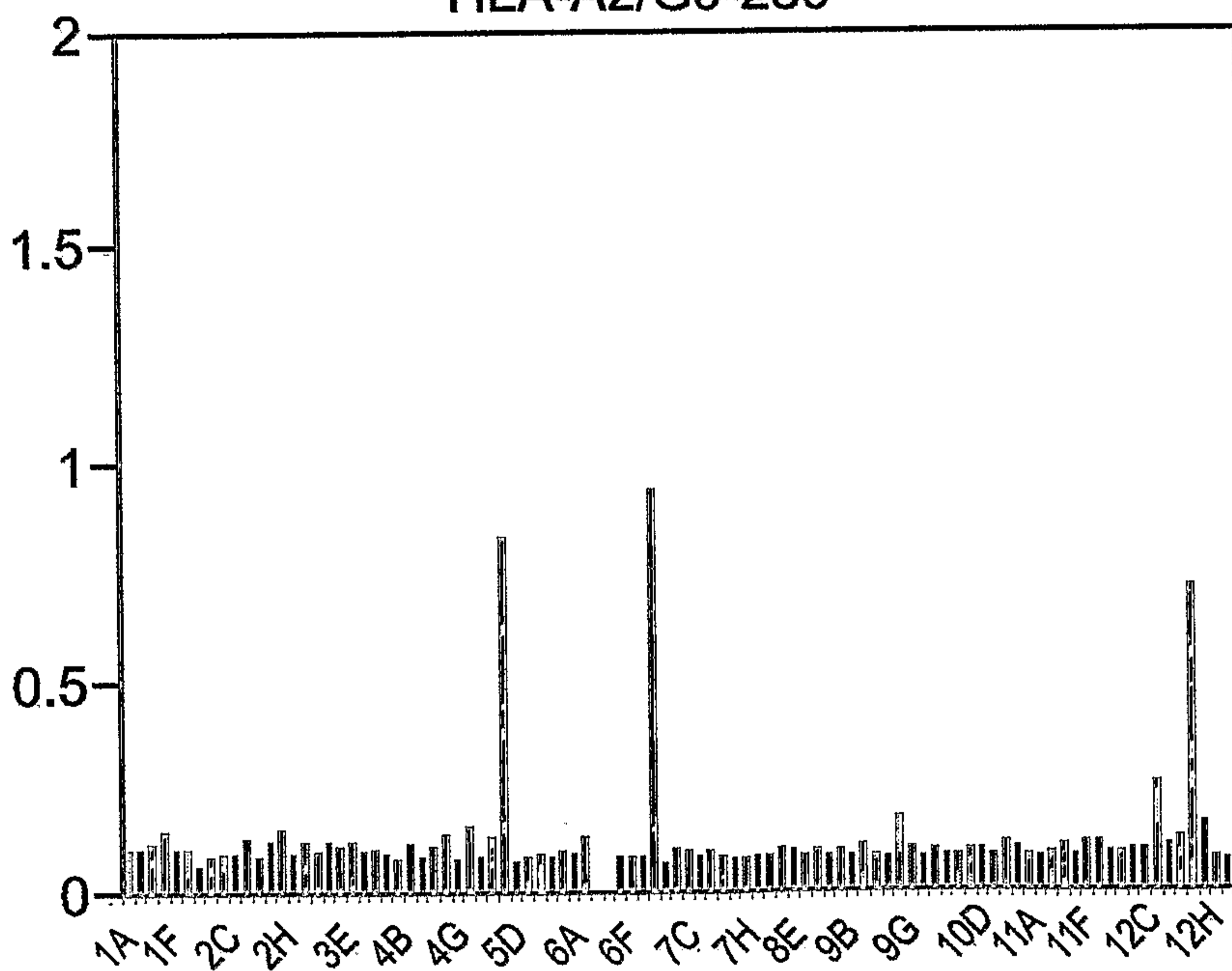


Fig. 2b



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 gln val lys leu gln glu ser gly gly gly leu val lys pro SEQ ID NO:9

15 16 17 18 19 20 21 22 23 24 25 26 27 28  
 GGA GGG TCC CTG AAA CTC TCC TGT GCA GCC TCT GGA TTC ACT  
 gly gly ser leu lys leu ser cys ala ala ser gly phe thr

29 30 31 32 33 34 35 36 37 38 39 40 41 42  
 TTC AGT AGC TAT GGC ATG TCT TGG GTT CGC CAG ACT CCA GAC  
 phe ser ser tyr gly met ser trp val arg gln thr pro asp

43 44 45 46 47 48 49 50 51 52 53 54 55 56  
 AAG AGG CTG GAG TGG GTC GCA ACC ATT AGT AGT GGT GGT AGT  
 lys arg leu glu trp val ala thr ile ser ser gly gly ser

57 58 59 60 61 62 63 64 65 66 67 68 69 70  
 TAC ACC TAC TAT CCA GAC AGT GTG AAG GGG CGA TTC ACC ATC  
 tyr thr tyr tyr pro asp ser val lys gly arg phe thr ile

71 72 73 74 75 76 77 78 79 80 81 82 83 84  
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 ser arg asp asn ala lys asn thr leu tyr leu gln met ser

85 86 87 88 89 90 91 92 93 94 95 96 97 98  
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 ser leu lys ser glu asp thr ala met tyr tyr cys ala arg

99 100 101 102 103 104 105 106 107 108 109 110 111 112  
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113 114 115 116 117 118  
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1 2 3 4 5 6  
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7 8 9 10 11 12 13 14 15 16 17 18 19 20  
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21 22 23 24 25 26 27 28 29 30 31 32 33 34  
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 met thr cys ser ala ser ser ser ile arg tyr ile tyr trp

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49 50 51 52 53 54 55 56 57 58 59 60 61 62  
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 asp thr ser asn val ala pro gly val pro phe arg phe ser

Fig. 3a

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63	64	65	66	67	68	69	70	71	72	73	74	75	76
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gly	ser	gly	ser	gly	thr	ser	tyr	ser	leu	thr	ile	asn	arg
77	78	79	80	81	82	83	84	85	86	87	88	89	90
ATG	GAG	GCT	GAG	GAT	GCT	GCC	ACT	TAT	TAC	TGC	CAG	GAG	TGG
met	glu	ala	glu	asp	ala	ala	thr	tyr	tyr	cys	gln	glu	trp
91	92	93	94	95	96	97	98	99	100	101	102	103	
AGT	GGT	TAT	CCG	TAC	ACG	TTC	GGA	GGG	GGG	ACA	AAG	TTG	
ser	gly	tyr	pro	tyr	thr	phe	gly	gly	gly	thr	lys	leu	

Fig. 3a (Cont.)

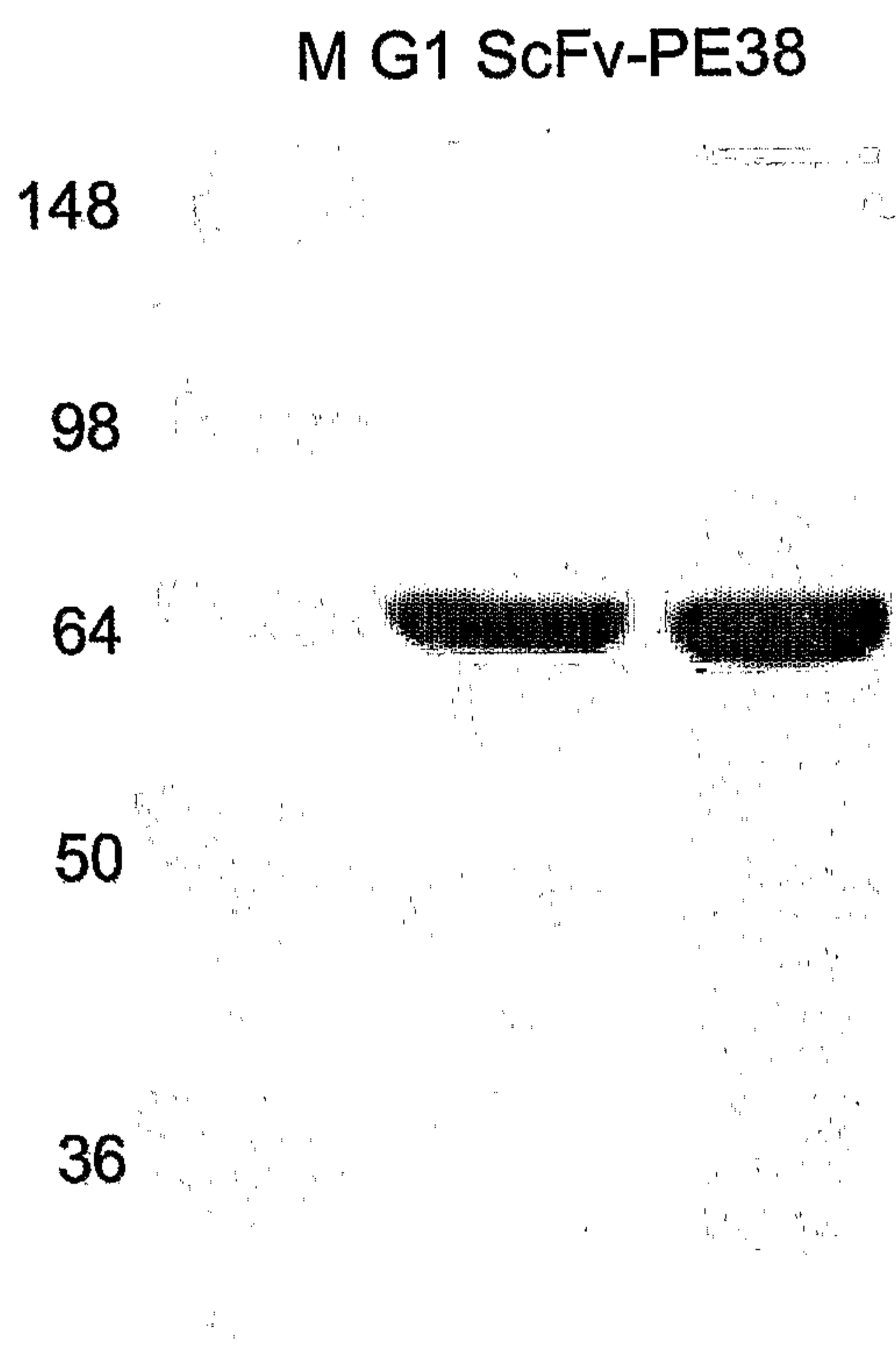


Fig. 3b

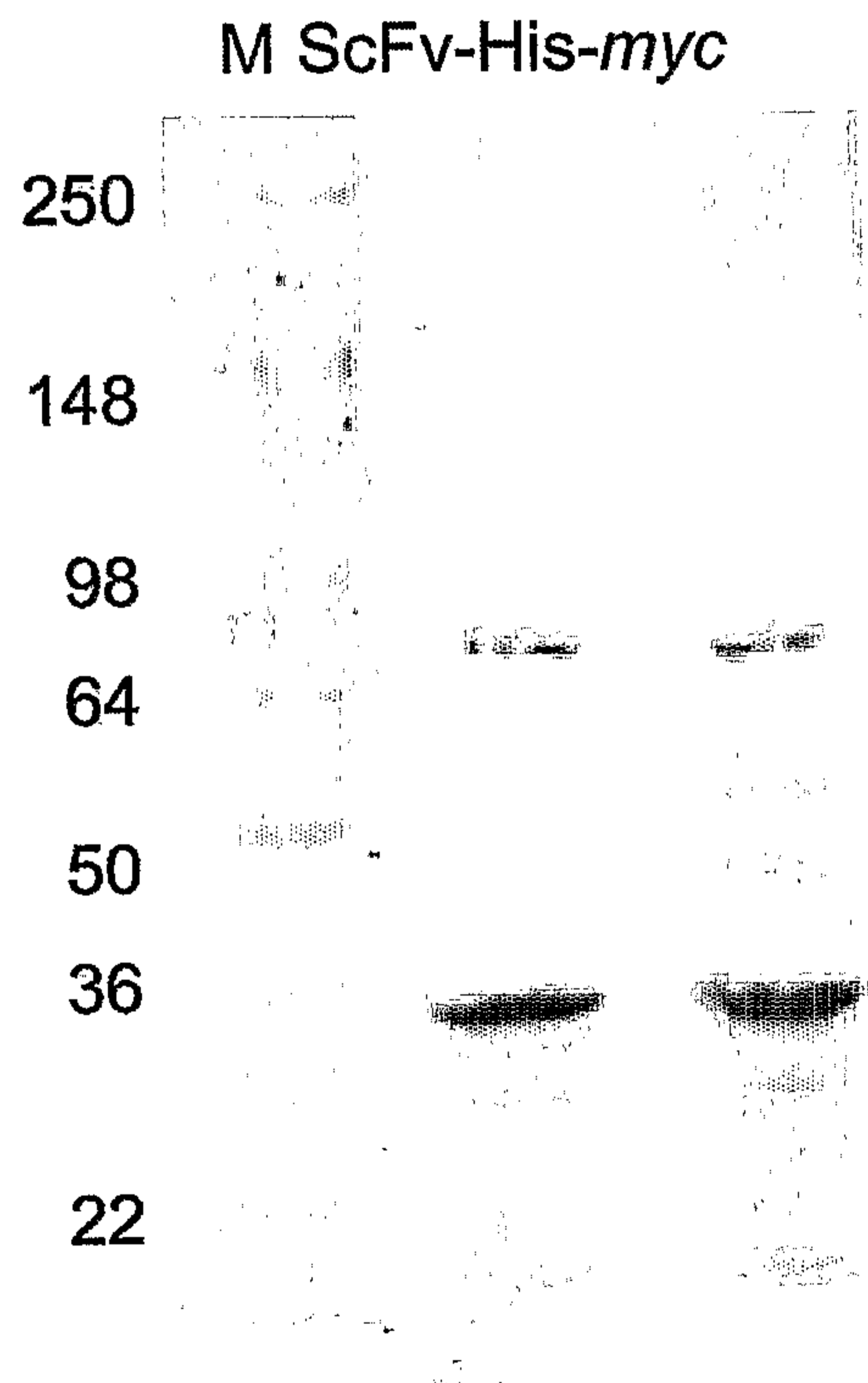


Fig. 3c



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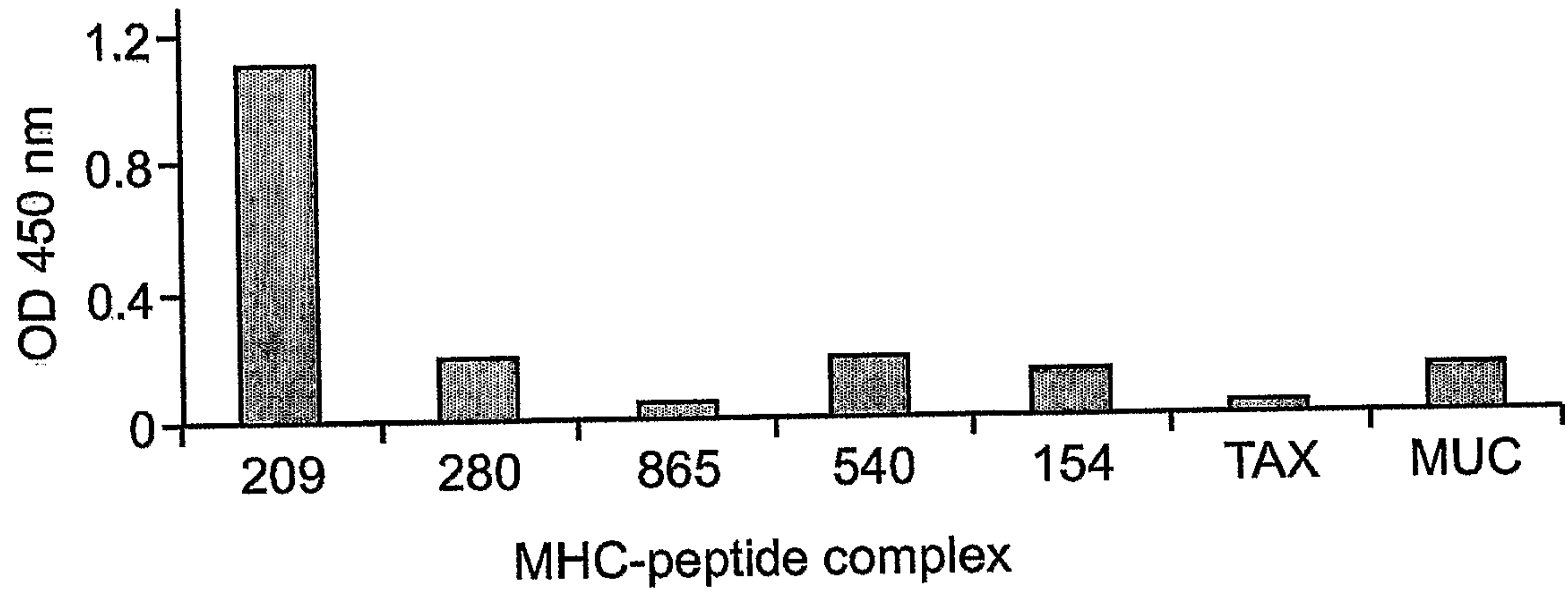


Fig. 4

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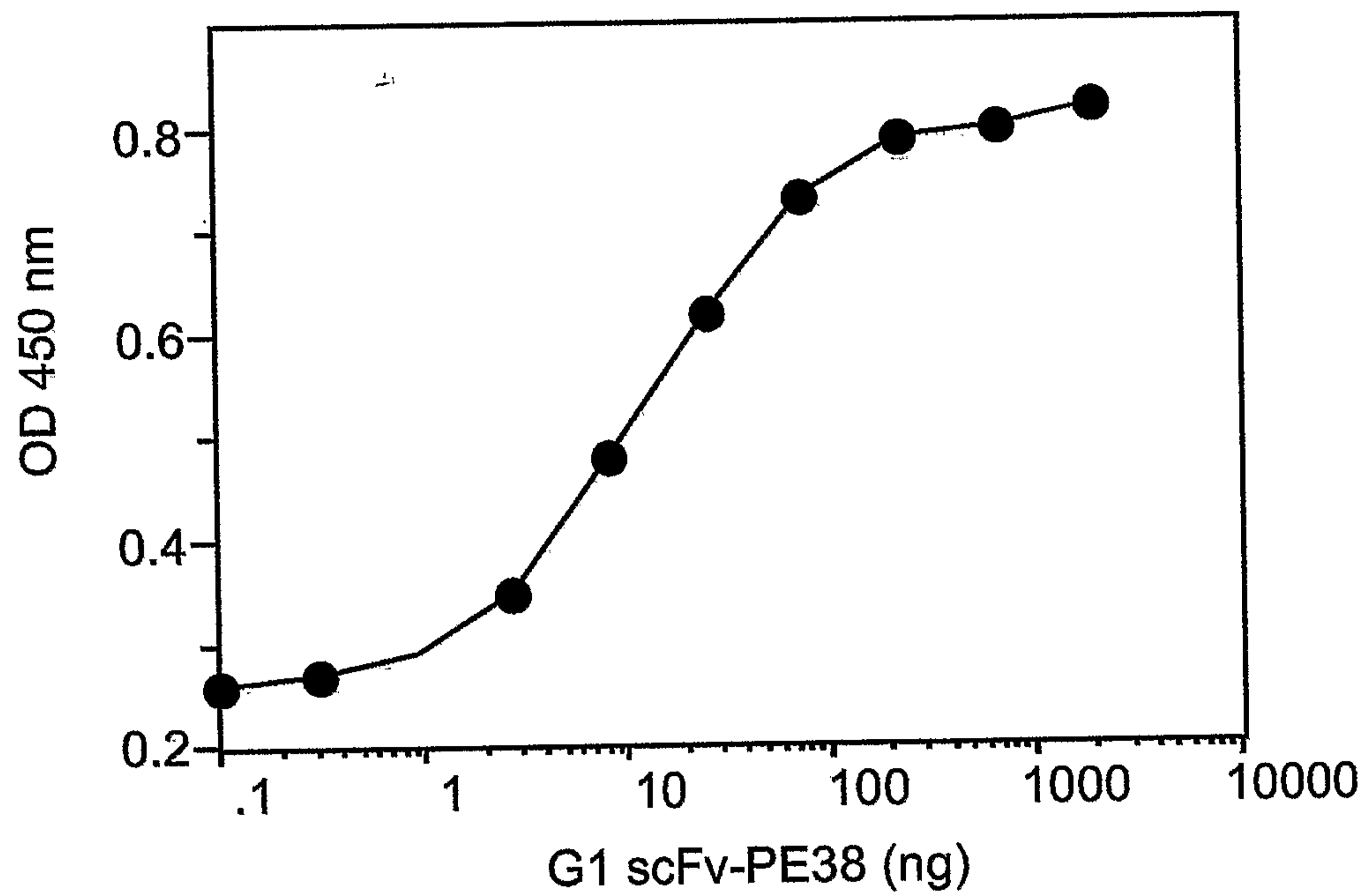


Fig. 5a

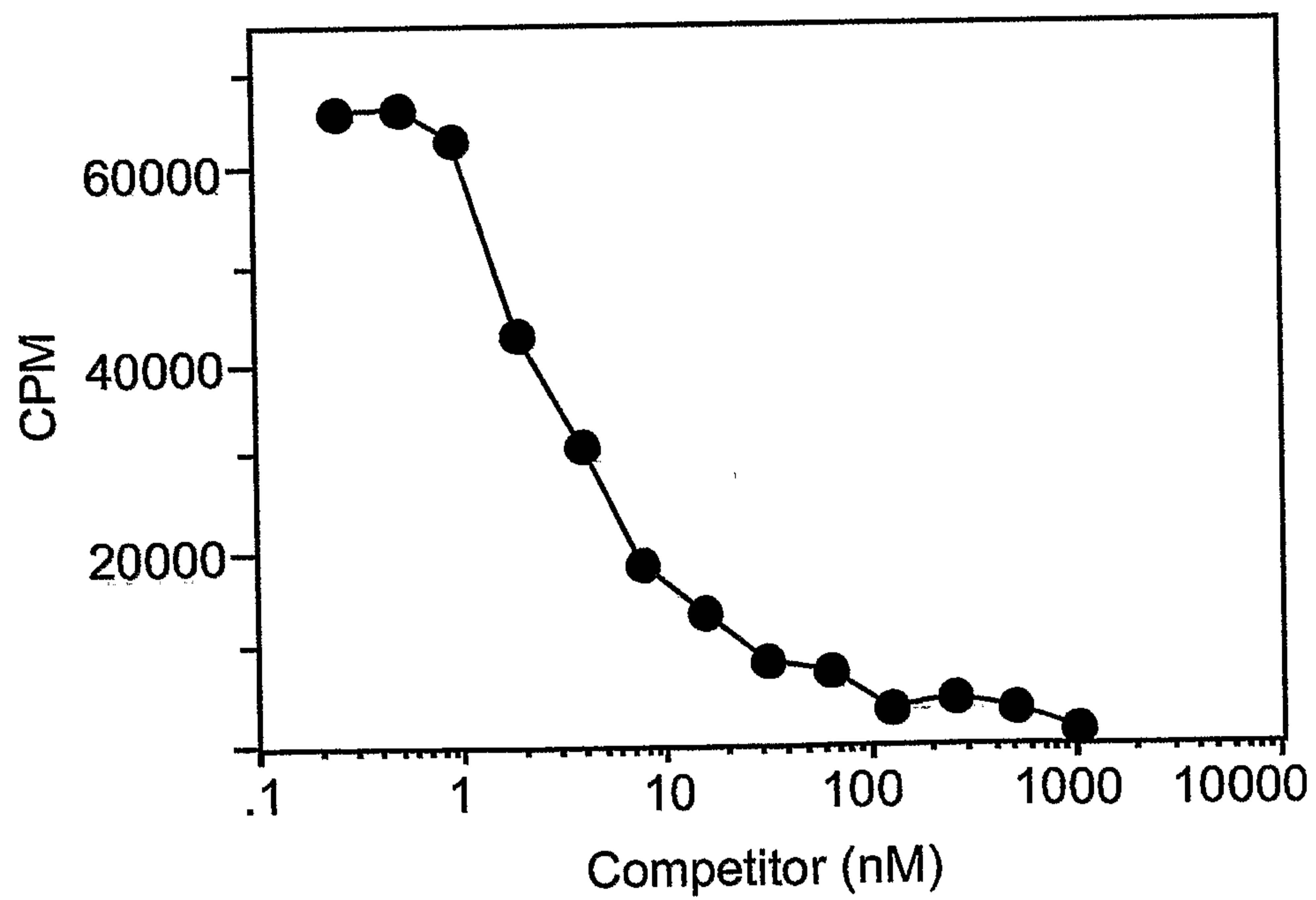


Fig. 5b



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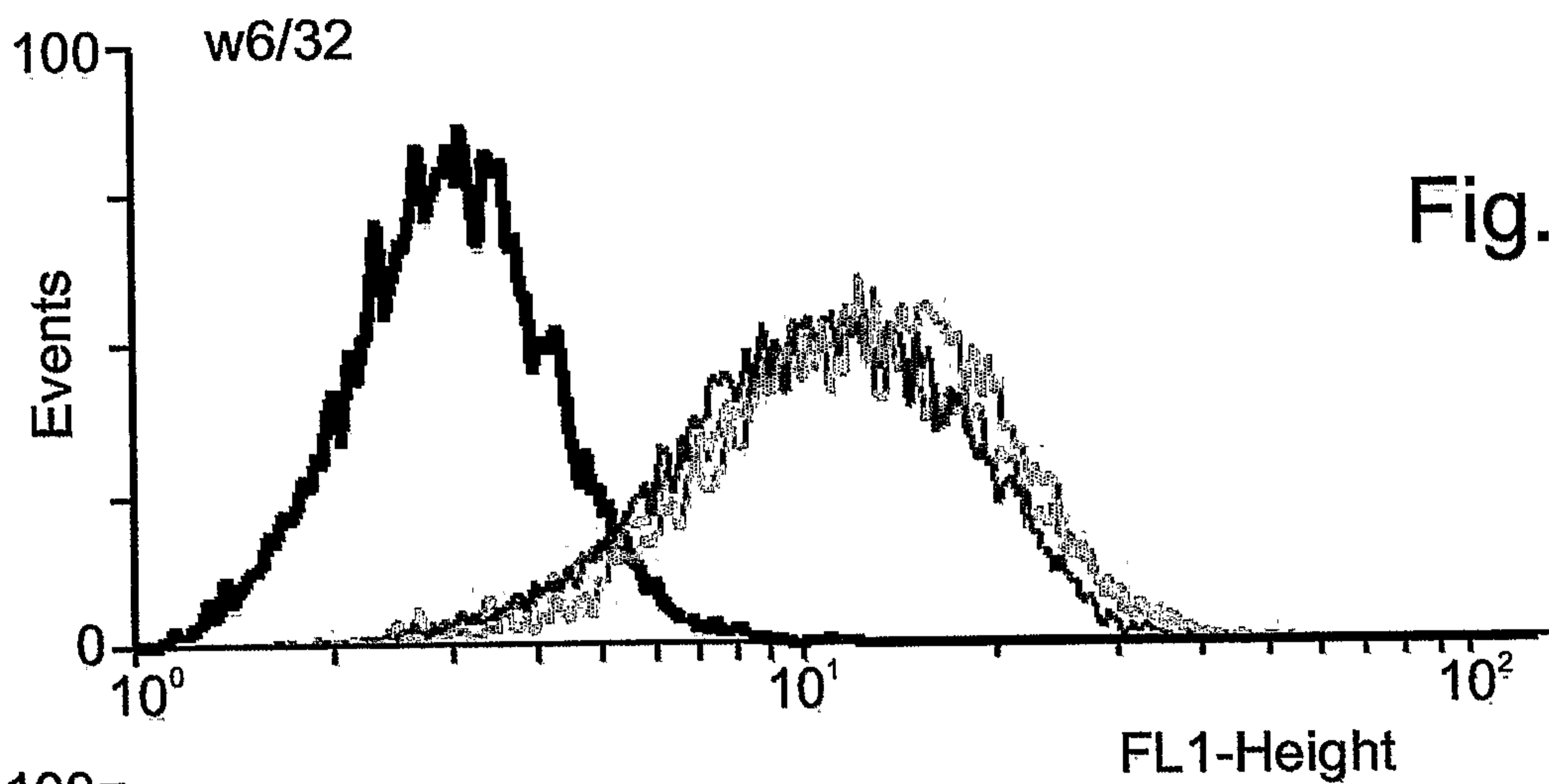


Fig. 6a

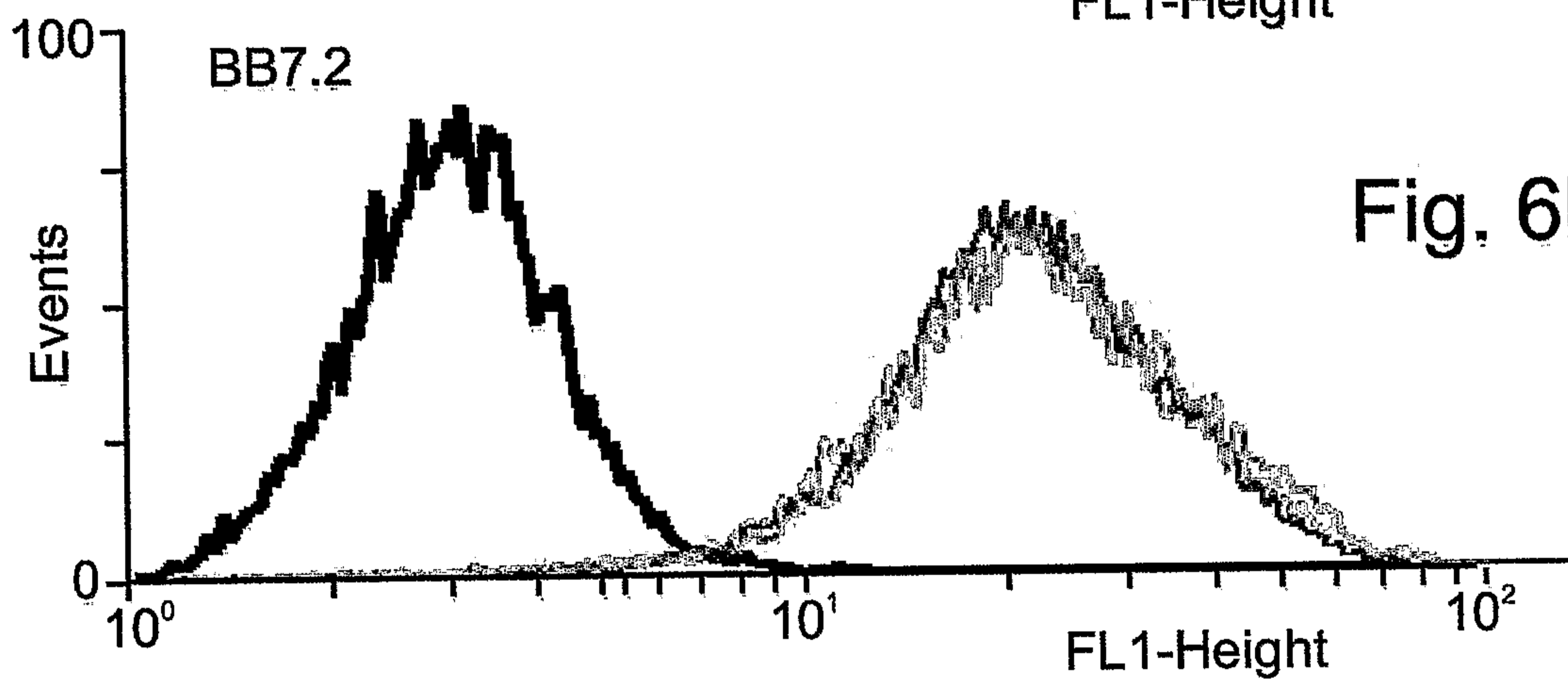


Fig. 6b

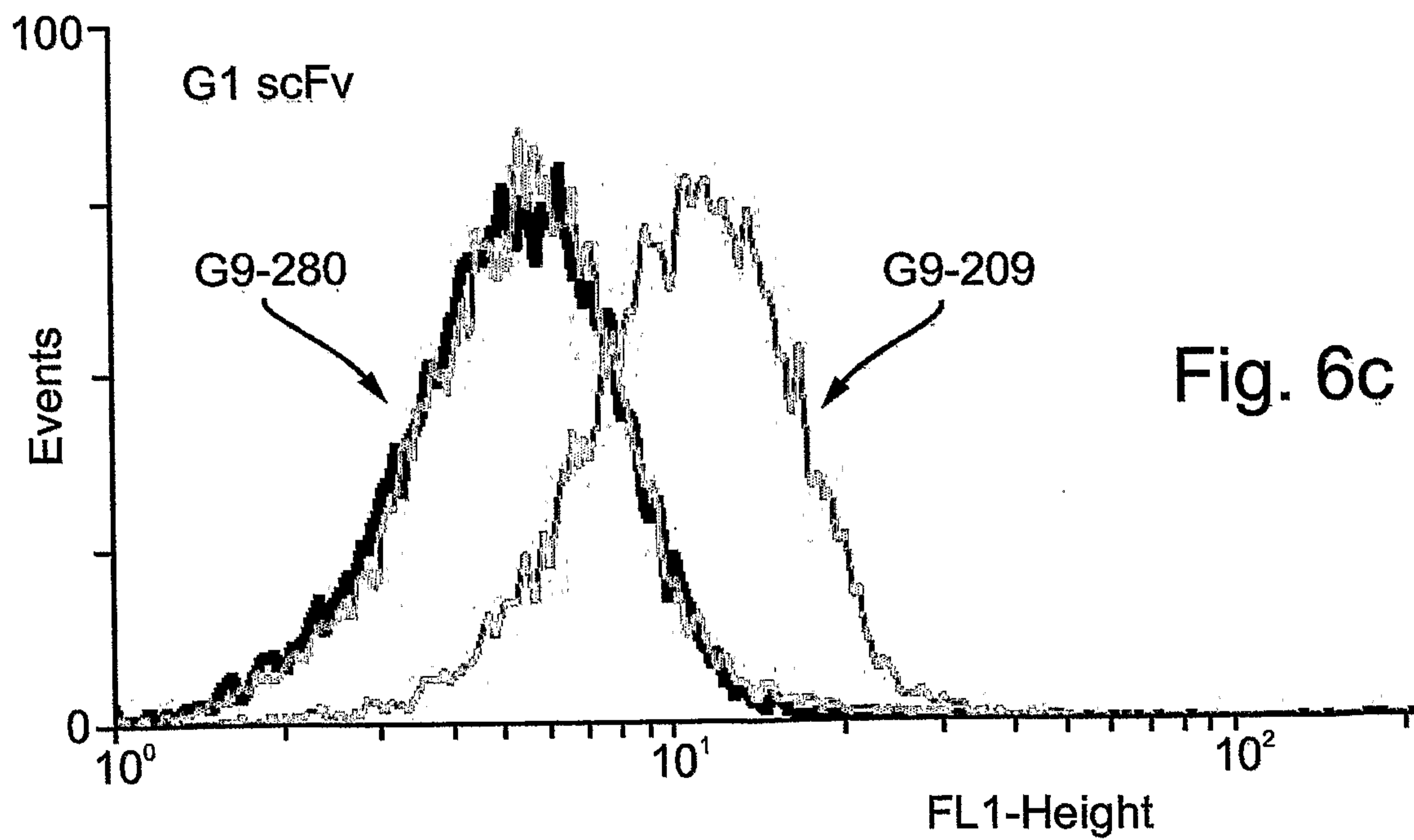


Fig. 6c

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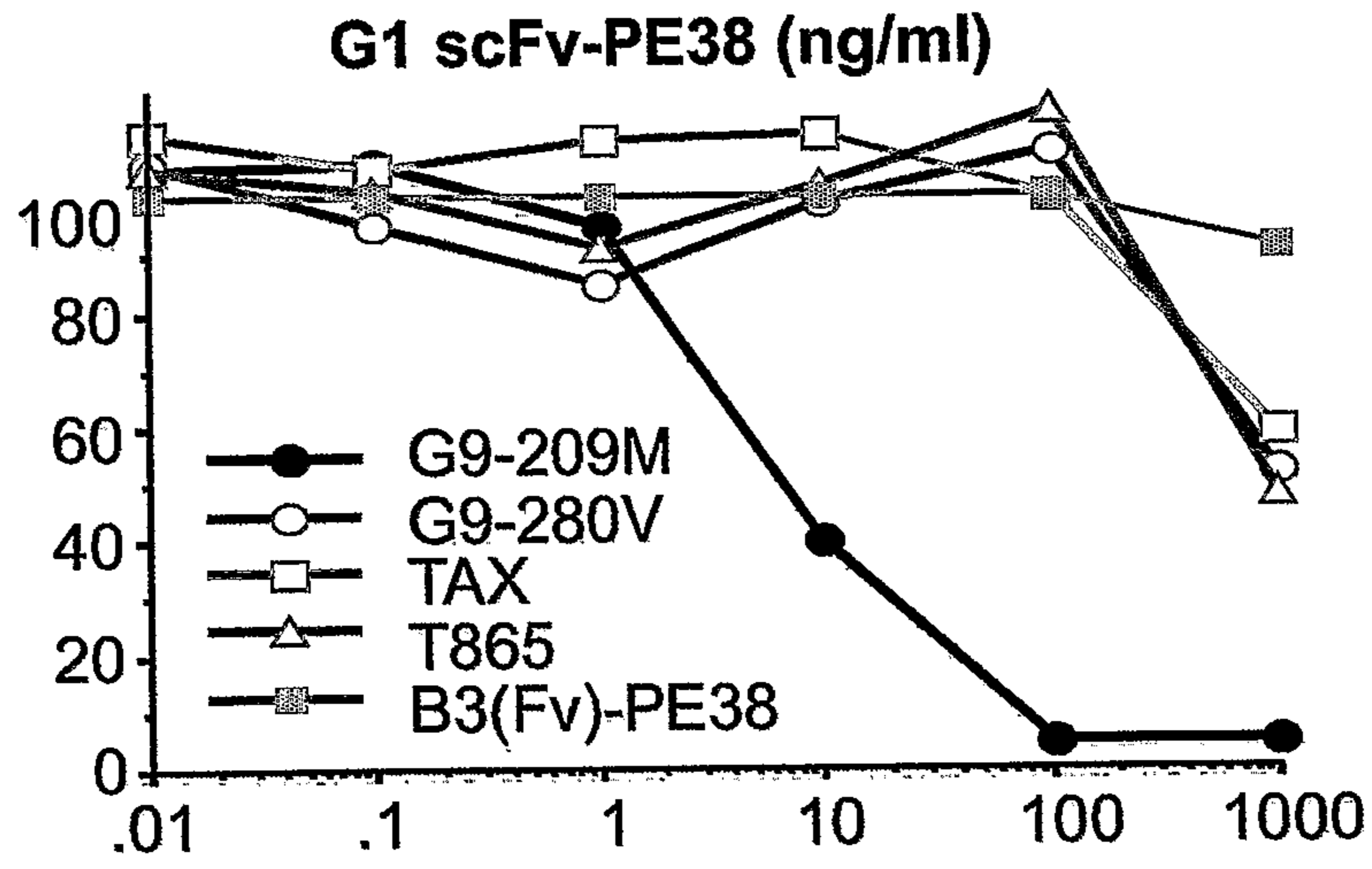
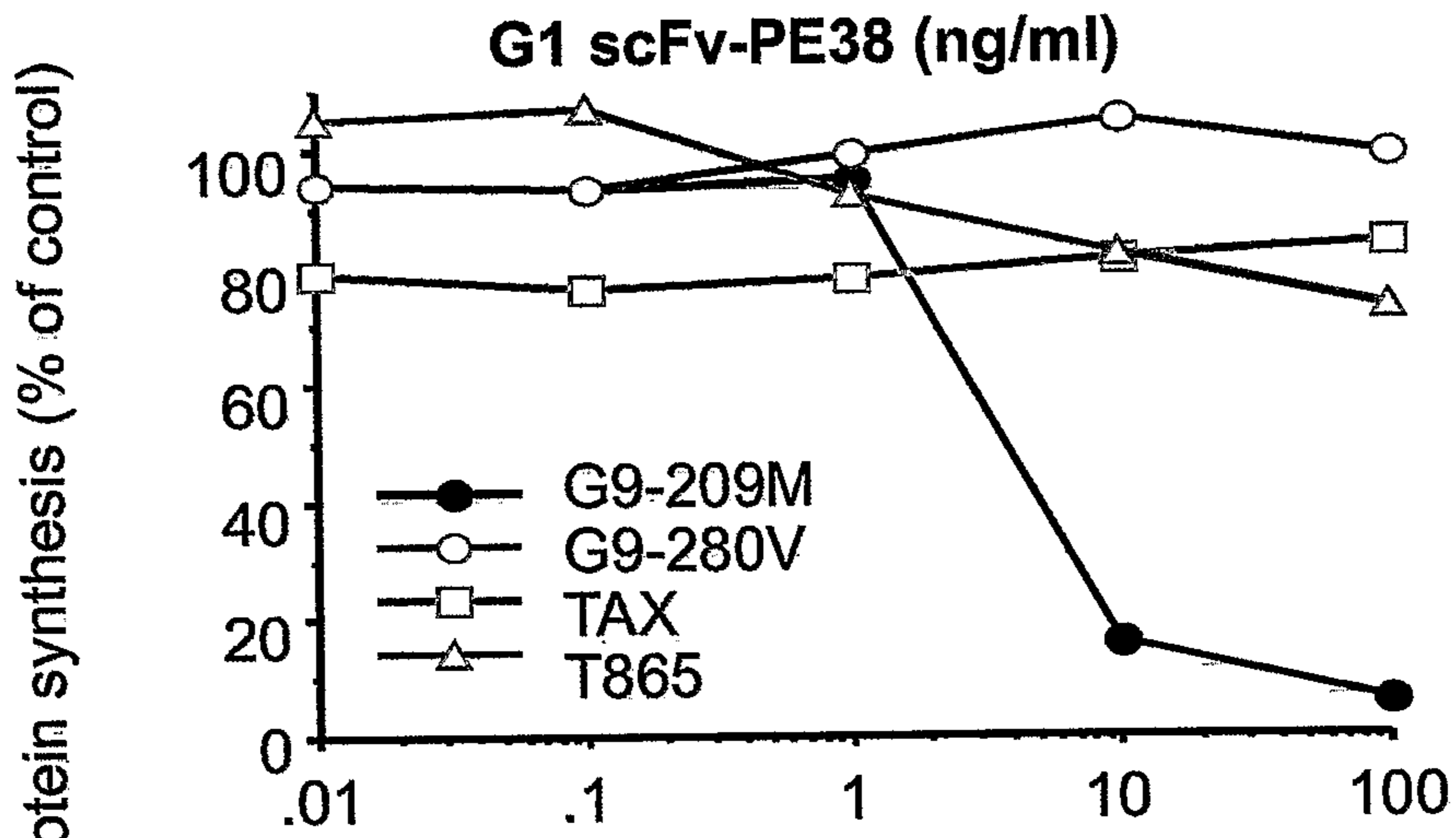
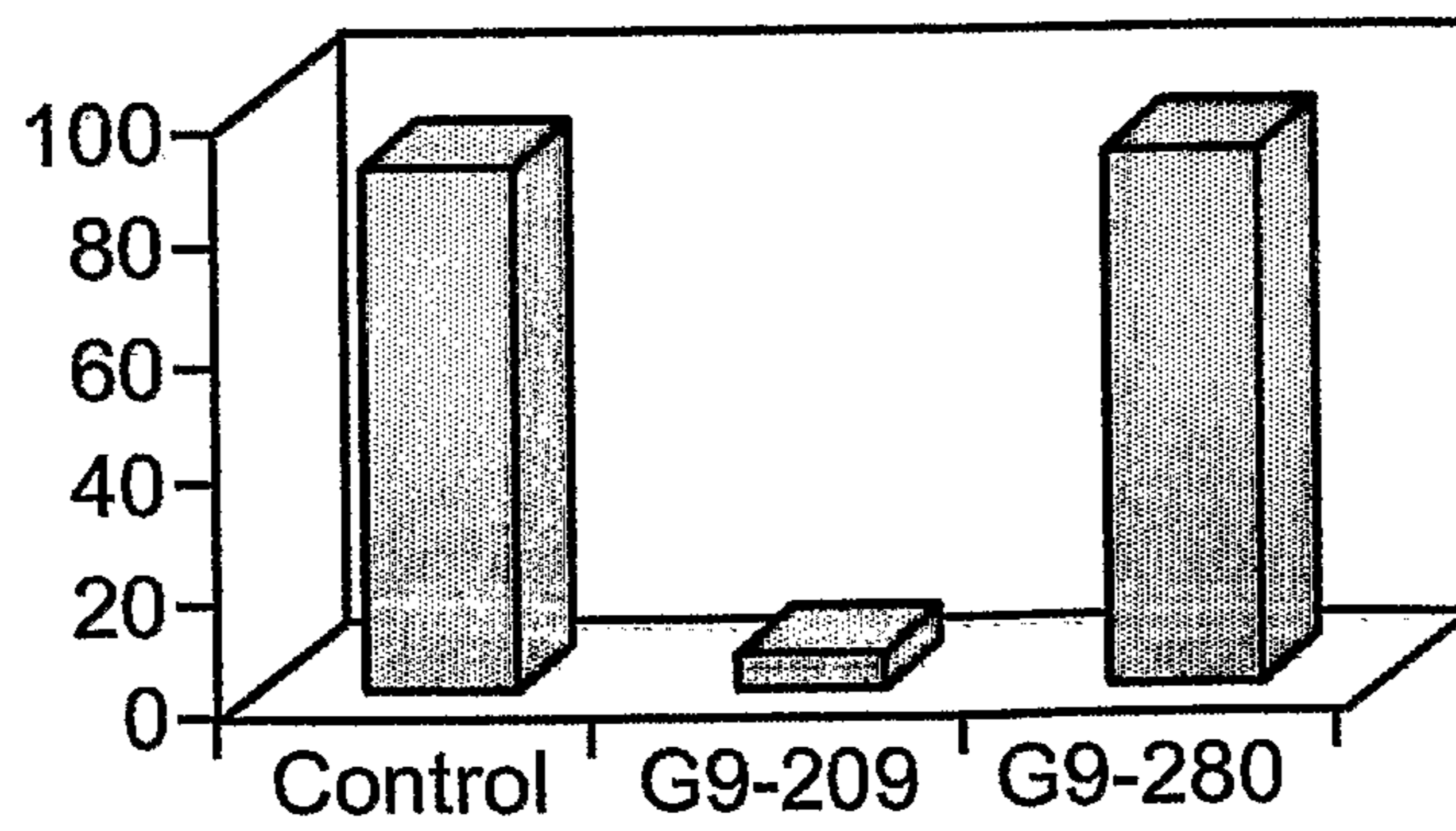


Fig. 7a



Protein synthesis (% of control)

Fig. 7b



Competitor

Fig. 7c